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THE EDITOR'S VIEWPOINT

A great deal has been said and written about the stability, or lack of stability, of aqueous solutions of sodium fluoride, and some unsupported statements have been used to promote the sale of certain types of fluoride solutions. Savchuck and Armstrong (p. 4) investigated the changes in aqueous sodium fluoride solutions of approximately 2 per cent strength stored in borosilicate glass flasks and in lime glassware. They believe that the effect of storage in the dark or on the laboratory shelf, has inconsequential effects upon the stability of 2 per cent sodium fluoride solutions. It would appear that if the clinician wishes to reduce the possible sedimentation in storage, storage in Pyrex would be preferred to so-called lime glass for containers but that there need be no alarm about the change of the strength of aqueous sodium fluoride solutions.

Shpuntoff and Rosebury (p. 7) have studied the infectivity of exudates from fusospirochetal lesions for guinea pigs, hamster, mice, and chick embryos. The material used was derived from a case of chronic suppurative periodontoclasia and had been passed through guinea pigs at least 14 times and stored by freezing. The guinea pig was infected by subcutaneous or intraperitoneal routes, the hamster, subcutaneously, and the mouse, either intraperitoneally or intracerebrally. However, the infectivity was uniformly low. Studies such as this should be carefully considered by the few clinicians who make the loose statement that bacteria have no role in the production of Vincent's infection.

Irving's study of the effect of fluorine on dentin in calcification in rats with low calcium rickets is reported, beginning on page 17. Single injections of F were used to obtain calciotraumatic effects. The dentin on the enamel side calcified up to the experimental line, but on the pulpal side a fine zone of matrix remained uncalcified, and then matrix calcified normally. The predentin in existence at the time of injection calcified completely. The reaction is quite different from that to F in normal animals or animals with high Ca rickets. The reason has not been found. It is possible that calcium levels may have a profound effect on fluoride reactions. This should be considered when fluoride therapy is suggested for children with developing teeth.

Dreizen, Greene, Carson, and Spies (p. 26) produced a yellow-brown melanin-like pigment in the organic matrix of noncarious human teeth crowns, in vitro, by the use of methylglyoxal and acetol. The claim had been made that pigmentation in dental caries is produced by microorganisms and is a sign of the carious process. This study shows that pigment can be formed by the interaction of the organic action of the enamel and dentin with carbonyl-containing products of carbohydrate degradation, and readily explains the presence of a yellow-pigmentation in a carious tooth without discarding the acid decalcification theory of dental caries. Further studies of this sort are necessary to add to the total knowledge of dental caries and to repute some of the claims that have been made on very tenuous evidence. "Failure to comprehend any of the biochemical aspects of caries" strongly limits the development of several "new" theories of dental caries, to paraphrase Leicester (Biochemistry of the Teeth, 1948, p. 272).

Scott, Kaplan, and Wyckoff, of the National Institute of Health, have studied the smooth surfaces of teeth by the means of metal-shadowed collodion replicas and have made observations on changes in those surfaces with age (p. 31). They found that the perikymata, enamel rod ends, and striations decreased with advancing age groups, but that they increased at a lower rate on proximal surfaces than on the buccal, lingual, and labial surfaces as might be expected because of the protection of the proximal surfaces from ordinary wear. The number, distribution, and character of crack-like structures, (lamellae?), however, do not appear to vary with age. The authors observed structures which may represent persistent areas of cuticle on the tooth surfaces of young and old individuals, and they

suggest that these structures may be important in view of the use of chemical agents for the control of dental caries. The authors warn of the danger of interpreting electron micrographs of tooth surfaces before adequate preliminary studies have been made at lower magnifications. This relatively new tool for the study of surface changes offers another method for advancing our knowledge of tooth structure and the pathology of tooth surfaces. It is hoped that studies of this nature will be continued by individuals who have developed the background, so that proper interpretations may be made and that premature applications of the method will not be brought forth by those who are not thoroughly acquainted with its shortcomings.

Ziskin, Applebaum, and Gorlin (p. 48) performed hypophysectomies on five Rhesus monkeys. A sixth monkey upon which an attempted hypophysectomy was unsuccessful served as an operative control. In studying the effect of removal of all or part of the hypophysis on the permanent dentition, they found the rate of dentin apposition in the Rhesus to be four microns per day but that the rate of growth differed in the various parts of the tooth. The calcification rhythm was about 16 microns per day. Hypophysectomy caused severe retardation in dentin apposition and interfered with calcification rhythm, but the calcification of enamel and dentin were of normal density.

Bruckner (p. 55) has investigated the presence of alkaline phosphatase in the pulpal tissues of human teeth. Previous investigators demonstrated the presence of this enzyme in the teeth of various mammals other than man. The report of Bruckner confirms earlier findings and extends this knowledge to the human teeth. Bruckner has also shown that the alkaline phosphatase can be demonstrated in the pulpal tissue of young teeth which have been decalcified in the presence of zinc ions under exacting conditions. The role of phosphatase in the calcification of cartilage and bone has not been fully evaluated. Some investigators have postulated possible mechanisms of calcification, but the complete story still requires further investigation. Histochemical methods are gradually extending our knowledge and it is highly important that these investigations be carried out for dental tissues as well as for other tissues of the body.

Sobel, Hanok, Kirshner, and Fankuchen (p. 61) have studied, chemically and by x-ray defraction, dentin and enamel of teeth, before and after shaking for 16 hours with a saturated fluoride solution, and with distilled water. They found similar apatite patterns in the enamel and dentin although wide variations in composition were noted. Phosphate was lost in preference to carbonate under the fluoride treatment, at a pH of 7.1. They also found that the calcium-phosphorus ratios of human dentin was usually higher than that of rat dentin and that the range of calcium phosphorus ratios of human enamel was smaller than that of rat enamel.

Dentay and Rae (p. 68) investigated the phosphate in saliva. Their chemical studies of filtered saliva showed no phosphatase activity and they, therefore, assume that saliva, as secreted by the glands, contains no phosphatase and suggest that "salivary phosphatase" is an improper term. They believe that the phosphatase in saliva is probably derived from cellular debris and food residue with a small contribution of microorganisms. Perhaps careful studies of other enzymes, which have been reported in the saliva, may show that they are contributions from extrasalivary material rather than from the secretions of the salivary glands.

Anderson (p. 72) in a study of the factors influencing CO_2 content of saliva found that unstimulated saliva loses CO_2 more rapidly than stimulated saliva. Anderson thought the explanation for the difference in behavior of stimulated and unstimulated saliva might be on the basis of the differences of pH of resting and stimulated saliva or on the presence of carbonic anhydrase in the saliva. The present study indicates that the differences in rate of CO_2 evolution are not the result of the presence of an enzyme in the saliva. Further studies have been carried out on the relationship between salivary pH and CO_2 evolution but are not reported in the present paper.

Granados, Glavind, and Dam (p. 77) fed two groups of hamsters two different types of diet in order to study the effect of diets having the same initial pH and the same

growth-promoting action but differences in quantity and quality of protein, fat, and carbohydrate. Seventy per cent of the two diets were supplied as identical ingredients, mostly carbohydrates. In Diet one, the carbohydrates were almost excluded from the variable 30 per cent, while Diet two had an additional 7.42 per cent of carbohydrate in the form of lactose. In Diet one, the fat was mainly from 3 per cent lard, and in Diet two from 5.37 per cent butter fat. Diet one contained 23.6 per cent protein supplied by casein, yeast, and corn, whereas Diet two contained only 12.5 per cent protein supplied by powdered milk, corn, yeast, and alfalfa. Diet one induced lower caries activity and higher periodontal disturbances. The authors suggest that the difference in protein content in the two diets may have played an important role in the inverse relationships between caries and periodontal lesions in the two groups but do not rule out the possible effects of the fats and carbohydrates. The authors also note that the animals came from stock which had been raised on Purina Laboratory Chow with a fluorine content of about 37.9 p.p.m. and suggest that this high fluorine content could be, in part, responsible for the low caries susceptibility in the animals studied. This work shows the difficulty in limiting dietary variations to a single factor. Although the authors, in this particular study, had 70 per cent of the diet the same in both groups, there were a number of factors including carbohydrate content, butter fat content, and protein content of the two groups which must be considered in arriving at any conclusion.

Glickman, Turesky and Hill (p. 83) have determined the oxygen consumption in normal and inflamed human gingiva using the Warburg manometric technic. They found that the oxygen consumption of gingiva was measurable by this method and that the oxygen consumption was not markedly altered in the gingiva with slight inflammation and proliferative changes, but that there was a tendency toward increased oxygen consumption in instances of marked chronic inflammation and proliferation of connective tissue and epithelium. Decreased oxygen consumption was noticed in gingivae which showed degeneration and necrosis. The changes in chronic inflammation of the gingiva, are comparable with those in kidneys, tendon, and skin.

The publisher of the *Journal of Dental Research* has been attempting to interest undergraduates in dentistry in the *Journal of Dental Research* by offering subscriptions to students at half rate. In addition to this, they are awarding scholarship subscriptions to the *J. D. Res.* through various schools of dentistry. The following twenty-seven men, who are recent graduates in dentistry, have been awarded such scholarships to the *Journal of Dental Research*. It will be interesting to notice how many of these recent graduates will continue their interest in either an active or passive way in dental research.

Dr. Basil W. Gibbs, Dr. Joseph G. Lebenzon, Dr. Irwin L. Marcus, Dr. John Ziegler, Dr. Kay H. Clifford, Dr. Lowell G. Larson, Dr. Joseph C. Muhler, Dr. Donald M. Williams, Dr. James Blake Adams, Dr. William R. Biddington, Dr. Mitchell J. Abdonore, Dr. Paul G. Zamzok, Dr. William S. Kramer, Dr. Donald C. Fitzroy, Dr. Paul Goldhaber, Dr. Bernard S. Schaffer, Dr. Irvin B. Prince, Dr. Frank E. Walter, Dr. Paul W. Montgomery, Dr. Ruth Durley Carter, Dr. Milton L. Westerberg, Dr. Fairfax Moody Hamilton, Dr. Calvin C. Cristensen, Dr. James E. Edson, Dr. William S. Takano, Dr. Louis J. Williams, Dr. William Gregorak.

H. B. G. R.

THE STABILITY OF AQUEOUS SOLUTIONS OF SODIUM FLUORIDE

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THE conclusive demonstration[1] of the effectiveness of topical fluoride application in reduction of caries has raised the question of the stability of the sodium fluoride solutions used, particularly with regard to changes in pH. The purpose of this investigation was to determine the action of light, dark, and atmospheric agencies, and of common types of glass containers on a 2 per cent sodium fluoride solution over a period of six months.

METHODS

A half-saturated (*circa* 2 per cent) solution of Baker's C.P. sodium fluoride was stored either in rubber-stoppered 200 ml. borosilicate glass (Pyrex) Erlenmeyer flasks or in 8 oz. oval Duraglas (presumably soda-lime-silica glass) prescription bottles with plastic screw caps. Containers for those solutions to be used in demonstrating atmospheric effects were left half full and were unstoppered at weekly intervals to permit displacement of the air within them. After gentle agitation they were restoppered and shaken for three minutes. All solutions not stored in the dark were kept together on the laboratory shelf.

Sodium was determined by means of a flame photometer,† and pH was measured to 0.05 pH unit with the glass electrode standardized with the potassium acid phthalate‡ buffer. Fluoride was titrated by the Armstrong[2] modification of the Willard-Winter[3] method. In the presence of sodium alizarin sulfonate the fluoride which is precipitated by thorium in this method produces a pink end point. Thorium nitrate is added very rapidly and continuously to the unknown until the appearance of a reddish-pink color similar to that of a prepared reference solution. Upon vigorous shaking the reddish-pink color fades into a faint pink, and the further addition of only a few drops of thorium nitrate develops a persistent color.

RESULTS AND DISCUSSION

The results are given in Table I. The gross appearance of the solutions in all the Pyrex containers after the six-month period remained unchanged. Surface films, dulling of the glass, and sediment were all absent. However, in the

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†Kindly supplied on loan by Dr. J. W. Berry of the American Cyanamid Co., Stamford, Conn.

‡Obtained from the Bureau of Standards, Washington, D. C.

TABLE I

CHANGES IN pH, SODIUM, AND FLUORIDE CONTENT OF SODIUM FLUORIDE SOLUTIONS STORED IN PYREX AND "LIME" GLASS CONTAINERS

	SAMPLE	INITIAL VOLUME OF SOLUTION	pH	Na^+ MG. PER 100 ML.	F^- MG. PER 100 ML.	Na/F WEIGHT RATIO	GROSS APPEARANCE
Original solution*			6.9	1093	900.	1.21	
Distilled water			6.15				
Theoretical value (22.9/19.00)						1.21	
FOLLOWING 6 MO. PERIOD:							
"Lime" glass							
Stored in dark	A1a	200 ml.	9.39	1083	901.4	1.20	Glass above solution dulled; surface film
	A1b	200 ml.	9.40	1083	900.8	1.20	Glass above solution dulled; surface film
	A1c	200 ml.	9.41	1070	910.7	1.17	Glass above solution dulled; surface film
Stored in light	A2a	200 ml.	9.46	1070	910.7	1.17	Glass above solution dulled; surface film
	A2b	200 ml.	9.41	1063	882.5	1.20	Glass above solution dulled; surface film
	A2c	200 ml.	9.47	1063	906.9	1.17	Glass above solution dulled; surface film
Weekly atmospheric exposure	A3a	100 ml.	9.47	1070	911.1	1.17	Glass above solution dulled; sediment (+)
	A3d	100 ml.	9.59	1070	921.8	1.16	Glass above solution dulled; sediment (+)
	A3b	100 ml.	10.10	1058	904.2	1.17	Glass above solution dulled; sediment (++++)
	A3c	100 ml.	10.11	1063	896.2	1.18	Glass above solution dulled; sediment (++++)
Borosilicate glass							
Stored in dark	B1a	200 ml.	7.51	1070	898.0	1.19	Unchanged
	B1b	200 ml.	7.57	1075	896.0	1.19	Unchanged
	B1c	200 ml.	7.56	1063	905.0	1.17	Unchanged
Stored in light	B2a	200 ml.	7.32	1075	908.1	1.18	Unchanged
	B2b	200 ml.	7.43	1075	916.9	1.17	Unchanged
	B2c	200 ml.	7.30	1088	908.7	1.20	Unchanged
Weekly atmospheric exposure	B3a	100 ml.	8.01	1093	921.6	1.19	Unchanged
	B3b	100 ml.	8.16	1088	909.1	1.20	Unchanged
	B3c	100 ml.	8.18	1095	908.3	1.21	Unchanged
	B3d	100 ml.	8.32	1093	895.2	1.22	Unchanged

*The original solution half-saturated with NaF containing by analysis 1093 mg. Na/100 ml. and 900 mg. F/100 ml. was thus a 1.99 per cent NaF solution.

soft glass bottles a film was present on the surface of all undisturbed solutions, and the glass above each solution was dulled. Sediments were present in each of the prescription bottles opened weekly.

The pH in all cases was alkaline, but significantly less so in the case of the solutions stored in Pyrex. Containers unstoppered weekly were uniformly of higher pH than those of the same series stoppered throughout the entire six-month period. There is no apparent correlation between pH and the weight ratio of Na/F. A possible explanation for the relatively greater decrement in the Na/F ratio in the "lime" glass series may be the formation of a sodium carbonate deposit on the inner surface of the prescription bottles. No significant differences could be detected whether the solution was stored in the dark or in the light for the period of the investigation.

Borosilicate (Pyrex) glass would appear to be relatively more resistant to the corrosive effect of fluoride solutions. The durability of the soda-lime-silica glass toward chemical reagents is of a lesser degree, whatever the mechanism of action of aqueous sodium fluoride upon the complex glass structure. Adsorption, leaching, hydrolysis, and subsequent disintegration of the "lime" glass by water and carbon dioxide within the bottle may possibly account for the dulling effect.

SUMMARY AND CONCLUSIONS

Storage of aqueous sodium fluoride solutions of approximately 2 per cent in stoppered borosilicate glass flasks is apparently without significant effect upon either the solution or the vessel over a six-month period. By contrast a dulling of the inner wall of the container and an increased alkalinity result from the use of "lime" glass prescription ware. Sedimentation occurs in those solutions in soft glass containers exposed frequently to the atmosphere. The pH's of all solutions stored in Pyrex show a consistently lesser increment in alkalinity than pH's of solutions contained in "lime" glass. The effect of storage in the dark or on the laboratory shelf upon the stability of a 2 per cent sodium fluoride solution seems inconsequential.

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INFECTIVITY OF FUSO-SPIROCHETAL EXUDATES FOR GUINEA PIGS, HAMSTERS, MICE AND CHICK EMBRYOS BY SEVERAL ROUTES OF INOCULATION

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PREVIOUS investigators have reported the production of characteristic experimental lesions by inoculation of exudates from Vincent's and other fusospirochetal diseases in healthy animals of several species, including rabbits,[1-5] guinea pigs,[5-8] mice,[5, 9] hedgehogs,[10] monkeys,[11] and a chimpanzee.[12] Hamsters and chick embryos do not seem to have been tested, and no previous attempt is known to have been made to determine the infectivity of these exudates by titration. The present investigation, part of a more extensive study of the bacteriology of fusospirochetal infections, includes roughly quantitative data on infectivity along with the results of efforts to determine which of the animals species tested is most satisfactory for titration and for maintenance of the infection by passage.

EXPERIMENTAL

Rabbits were not used in these tests after preliminary trials[13] had shown them to be excessively sensitive to the chance occurrence of hemolytic streptococci or other pathogens in gingival scrapings. The adult hosts used consisted of guinea pigs, hamsters, and mice, there being no indication in the literature of any advantage outweighing the obvious disadvantages of the other species mentioned above. The subcutaneous route of inoculation was used with guinea pigs and hamsters, the intraperitoneal route with guinea pigs and mice, and the intracerebral route with mice. Chick embryos were inoculated on the chorioallantoic membrane, into the yolk or amniotic sacs, or subcutaneously into the embryo. Dosage and other data are given below.

The fusospirochetal exudates used were obtained originally from a case of chronic suppurative periodontoclasia† as a heavy suspension of gingival scrapings in 1 ml. of extract broth. This suspension was inoculated subcutaneously into 2 guinea pigs and the resulting whole exudates were then passed subcu-

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taneously at least 14 times through guinea pigs by the method described by Rosebury and Foley.[8] Deliberate traumatization during inoculation was not resorted to after the first passage. Hemmens and Harrison[14] have reported that these exudates acquire stable properties after seven such passages. Pus obtained from a guinea pig lesion, by aspiration with a syringe containing 1 ml. of extract broth, was frozen in CO_2 ice in sealed ampules. When thawed rapidly in a water bath at 37° C., after having been frozen for as long as 13 months, such exudates uniformly have shown the typical fusospirochetal flora by darkfield examination, with motility of spirochetes, vibrios, and other motile microorganisms apparently unimpaired. These thawed exudates have been regularly infective for guinea pigs in dosages of 0.1 to 0.3 ml. without dilution, yielding responses similar to those obtained with equal amounts of fresh exudates.

Titrations for infectivity were made by successive dilution of exudates in extract broth. Inoculated animals were examined daily, and animals that died or were sacrificed at intervals were autopsied and their exudates or fluids examined by darkfield examination.

Subcutaneous Inoculation of Guinea Pigs and Hamsters.—Six guinea pigs or 5 hamsters were inoculated subcutaneously with each of the following dilutions of exudate: 0, 1:5, 1:10, 1:20, 1:40, and 1:80. The results are summarized in Table I. The characteristics of spreading and localized lesions in guinea pigs were similar to those described by others,[3, 5, 8, 15, 16] with exceptions and additions to be noted. Exudates from all lesions showed the characteristic fusospirochetal microorganisms under darkfield.

TABLE I

EFFECTS OF SUBCUTANEOUS INOCULATION OF FUSOSPIROCHETAL EXUDATE IN GUINEA PIGS AND HAMSTERS

(Inoculum, 0.1 ml.)

DILUTION OF EXUDATE	GUINEA PIGS			HAMSTERS			
	FATAL EXTENSION	LOCALIZED, RECOVERED	NEGATIVE	FATAL EXTENSION	EXTENSION, RECOVERED	LOCALIZED, RECOVERED	NEGATIVE
	Number of Animals						
0	3	2	1	2	3	0	0
1:5	1	4	1	0	3	1	1
1:10	0	0	6	1	0	1	2*
1:20	0	0	6	0	0	0	5
1:40	0	0	6	0	0	0	5
1:80	0	0	6	0	0	0	5

*An additional hamster died with intercurrent infection, showing no evidence of fusospirochetal infection.

Results in Guinea Pigs.—No specific lesions were found in guinea pigs inoculated with 1:10 or higher dilutions of exudate. Of the guinea pigs with spreading lesions ("extension"), 1 died on the fourth day and 2 were sacrificed while moribund on the seventh day. One additional animal was lost on the

twelfth day, having shown a lesion similar to those of the spreading and fatal type. The localized lesions became evident within 3 days after inoculation. Two of the 6 animals with such lesions were sacrificed on the sixth day after inoculation, 1 died on the twenty-first day with an intercurrent pneumonia, and the others were allowed to recover.

The localized lesions in guinea pigs began as a red edematous area at the site of inoculation and developed into a nodule whose periphery increased in density while the center tended to liquefy, as in a typical abscess. Thereafter they tended to heal, in smaller lesions by resorption, in larger ones by evacuation. When evacuation occurred it followed the peripheral formation of fibrous tissue. Animals with well-localized lesions did not appear sick, and when sacrificed showed no abnormalities of the viscera. Even large localized lesions, which have contained as much as 50 ml. of pus, did not seem to interfere with function in the guinea pig.

Spreading lesions in guinea pigs were similar to local lesions initially, but failed to be walled off by fibrous tissue. Their margins remained indistinct, tapering off into the normal area. At autopsy the fascial planes were found to have been dissected by serofibrinous exudate at the advancing periphery of the lesion. There was usually a marked accumulation of thin foul exudate, usually greenish, and an abundant formation of fibrinous adhesions. At the site of inoculation marked capillary hemorrhage was the rule, coloring the area from dark red or greenish to black. In animals sacrificed while moribund, the internal organs were somewhat congested, and the adrenals were enlarged and hemorrhagic.

The spreading lesion tended to proceed anteriorly from the site of inoculation in the groin, usually remaining within the confines of the midventral and midlateral lines, but sometimes crossing one or both. Mapping of the areas of subcutaneous involvement in a series of 19 such animals indicated that death usually ensued when the process entered the axillary space, or otherwise when it had extended over approximately 20 or 30 per cent of the total body surface. This extensive involvement, accompanied by only slight visceral changes, suggests that death may have resulted from nonspecific toxemia due to massive tissue damage.

Results in Hamsters.—In hamsters, as is shown in Table I, no specific lesions appeared after inoculation of exudate dilutions of 1:20 or higher. Lesions tending to localization from the outset were found in only 2 animals. Of the 9 animals with spreading lesions only 3 died (5, 9, and 10 days after inoculation, respectively). In the others, late localization of the cellulitis occurred and the lesions evacuated and healed. Evacuation in these as in the two initially localized lesions occurred early in most animals, so that the large abscesses containing abundant fluid pus, which have been common in guinea pigs inoculated subcutaneously, were not characteristic of the hamster. Both localized abscesses and fatal spreading infections in the hamster, when they appeared, were similar to those in the guinea pig.

The hamster seems to be slightly more sensitive to subcutaneous fusospirochetal infection than the guinea pig, and it is evident from the data that there was a distinctly greater tendency to cellulitis in the hamster. On the other hand, spreading lesions, nearly always fatal in guinea pigs, eventuated in recovery in 6 of 9 hamsters. The skin of the hamster is relatively thin, and over an accumulation of pus, readily becomes ischemic and gangrenous, with resulting evacuation. Probably because of the loose areolar subcutaneous tissue of the hamster and the marked mobility of its skin, spreading tracts tend to form much more readily than in the guinea pig. On the other hand, the early evacuation of such tracts promotes healing rather than fatality in the hamster.

Intraperitoneal Inoculation of Guinea Pigs and Mice.—Exudate dilutions from 0 to 1:160 were used with 3 mice at each dilution level in the first experiment, from 1:5 to 1:320 with 5 mice each in the second, and from 1:5 to 1:160 with 3 guinea pigs each in the third. The exudate used as inoculum in the guinea pig series (sixteenth guinea pig passage) was also inoculated subcutaneously into 4 guinea pigs, using 0.1 ml. undiluted. All 4 of these animals showed characteristic responses.

Table II presents the results following intraperitoneal inoculation. Six mice in Experiment II and 3 guinea pigs showed localized abscesses. They were found in 1 mouse that received exudate diluted 1:160, and in 1 guinea pig given a dilution of 1:40. On the other hand, 2 mice and 1 guinea pig inoculated with a dilution as low as 1:5 showed no indication of infection. The local lesions appeared at the site of inoculation in the lower left quadrant, and involved omentum, peritoneal wall, and an intestinal loop, or occurred in the area of contact of liver, stomach, and spleen, involving any or all of these organs along with the omentum and an intestinal loop. These abscesses contained typical fusospirochetal exudates.

TABLE II

EFFECTS OF INTRAPERITONEAL INOCULATION OF FUSOSPIROCHETAL EXUDATE IN MICE AND GUINEA PIGS

(Inocula: Mice, 0.5 ml.; Guinea Pigs, 1.0 ml.)

	I. MICE		II. MICE			III. GUINEA PIGS		
DILUTION OF EXUDATE	GENERALIZED PERITONITIS	NEGATIVE	GENERALIZED PERITONITIS	LOCALIZED ABSCESS	NEGATIVE	GENERALIZED PERITONITIS	LOCALIZED ABSCESS	NEGATIVE
	Number of Animals							
0	3	0						
1:5	3	0	2	1	2	2	0	1
1:10	3	0	2	1	2	1	2	0
1:20	2	1	2	1	2	2	0	1
1:40	1	2	2	1	2	0	1*	2
1:80	1	2	0	1	4	0	0	3
1:160	0	3	0	1	4	0	0	3
1:320			0	0	5			
Broth control			0	0	5			

*Localized but fatal fusospirochetal infection. See text.

One of the 3 guinea pigs with localized lesions died 7 days after inoculation. In this animal the lesion, although localized, was very large, and there was extensive inguinal lymphadenitis. Typical fusospirochetal organisms were seen both in the abscess and in the affected lymph nodes.

In the local lesions the affected organs were glued together by a fibrinous or fibropurulent exudate infiltrated by adhesions. Localization seemed to depend on the greater omentum, which was intimately involved in the associated fibrosis. The lesions and their sites of localization were very similar in the two animal species.

Fatal peritonitis occurred in a total of 21 mice following inoculation of exudate dilutions as high as 1:80. A similar result developed in 5 guinea pigs, but in this species the highest effective dilution was 1:20. Since the volume of inoculum in these experiments was 5 or 10 times that used subcutaneously, the data may be said to show little difference in sensitivity to infection by the two routes; however, the mouse appears to be somewhat more sensitive than the guinea pig to intraperitoneal inoculation.

Both mice and guinea pigs with generalized peritonitis died in from 1 to 8 days. At autopsy a foul cream-colored or yellowish to greenish fibrinous exudate covered all the abdominal viscera. The greater omentum in particular was thickened and loosely adherent to the underlying intestines, and the abdominal blood vessels were markedly congested. Darkfield examination of the exudate revealed the typical fusospirochetal flora.

Intracerebral Inoculation of Mice.—In one experiment 3 mice, and in a second experiment 4 or 5 mice, were inoculated intracerebrally with 0.03 ml. of exudate dilution ranging from 0 to 1:160. The results are summarized in Table III. All but one of the mice that received dilutions up to 1:20 died within 3 days with generalized encephalitis. At autopsy there was either a massive suppurative involvement of the brain substance or an abscess containing gelatinous exudate, extending over one quarter or more of the brain volume. Exudates from mice inoculated at the 0 and 1:5 dilution levels showed the typical fusospirochetal flora under darkfield. In mice at the 1:10 and 1:20 levels, with one exception in the latter group (see Table III), no spirochetes were found; but the other members of the fusospirochetal flora, including fusiform bacilli, cocci, and vibrios, were all plentiful.

The mice listed as survivors in Table III showed no gross indication of infection. The 10 survivors in Experiment I were reinoculated intraperitoneally 17 days after intracerebral inoculation with 0.5 ml. of 0, 1:5, or 1:20 dilutions of exudate. A parallel group of previously uninoculated mice were treated similarly as controls. Equivalent responses in the two groups gave no clear evidence of immunity after survival from intracerebral inoculation. The 15 survivors of Experiment II (Table III) were sacrificed between the fifth and the

TABLE III

EFFECT OF INTRACEREBRAL INOCULATION OF FUSOSPIROCHETAL EXUDATE IN MICE

(Inoculum 0.03 ml.)

DILUTION	EXPERIMENT I		EXPERIMENT II	
	DIED WITH GENERALIZED ENCEPHALITIS	SURVIVED	DIED WITH GENERALIZED ENCEPHALITIS	SURVIVED
	Number of Mice			
0	3		5	
1:5	3		5	
1:10	2*	1	4*	
1:20	3†		3*	1
1:40		3	1*	4
1:80		3		5†
1:160		3		5

*Exudate from the brains of these animals did not show spirochetes.

†One animal in each of these groups showed a localized brain abscess with exudate positive for spirochetes.

eleventh day after intracerebral inoculation. All but one were negative. One animal showed a discrete abscess, about 2 mm. in diameter. Darkfield examination of the greenish exudate aspirated from this lesion showed the typical fusospirochetal flora by darkfield examination.

Inoculation of Chick Embryos.—Inoculation of 10-day-old embryonated eggs, with dilutions of fifteenth passage guinea pig exudate, on the chorioallantoic membrane or via yolk or amniotic sacs yielded irregular results. At the lower dilutions death of the embryo generally resulted in 3 days or less. In embryos alive on the fourth day, darkfield examination of fluids and membranes showed cocci, nonmotile bacilli, and fusiform rods in abundance, but so few spirochetes or other motile forms as to suggest lack of multiplication. Similar results were obtained with 11-, 14-, and 17-day embryos, and with subcutaneous inoculation of the embryo as well as with the other routes of inoculation. Attempts at successive passage through 3 series of embryos, stimulated by the reports of Hallauer and Kuhn[17] with *Treponema pallidum* and of Steinhaus and Hughes[18] with an unidentified blood spirochete, again resulted in progressive diminution of spirochetes. Minced tissue suspension from infected embryos of the first passage failed to reproduce fusospirochetal infection when inoculated subcutaneously into guinea pigs with or without deliberate traumatization.

More successful results were obtained following inoculation of fusospirochetal exudate mixed at 40° C. with nutrient agar. An equal volume of beef infusion containing 1 per cent agar and 1 per cent dextrose was mixed with fifteenth passage exudate, and 0.2, 0.1, or 0.05 ml. of the mixture inoculated into 10-day embryos via the amniotic sac. Of 8 embryos thus inoculated, 5 died within 6 days with nonspirochetal infection similar to that in previous series. In the other 3, sacrificed and examined on the sixth day, the chorioallantoic membrane was markedly thickened and the embryo was swollen, translucent,

and edematous, with its blood vessels congested especially in the extremities and brain. Darkfield examination of the extra-embryonic fluids and of the embryo was negative, but examination of ground suspensions of chorioallantoic membrane showed abundant spirochetes in an apparently complete fusospirochetal flora. Similar results were obtained in two successive replicates of this experiment, using 13-day and 10-day embryos; but an attempt at passage to fresh embryos, using as inoculum a darkfield-positive suspension of chorioallantoic membrane, was unsuccessful.

DISCUSSION

The maintenance of fusospirochetal exudates by storage in CO_2 ice (at –70 to –78° C.) without appreciable loss of either motility or infectivity for as long as 13 months indicates the value of this method, and suggests its usefulness for storage of pure cultures derived from such exudates. This method of storage is being used sucessfully for pure cultures in this laboratory and will be reported on in detail elsewhere. Hemmens and Harrison[14] stored fusospirochetal exudate at –70° C. for periods up to 24 days and noted "only slight loss of infectability and no quantitative changes in the flora." In our experience exudates after thawing have not only shown undiminished infectivity, but on darkfield examination have been indistinguishable from fresh exudates. The normal appearance and active motility of the spirochetes immediately after thawing contrasts with the distortion and immobility usually shown by relapsing fever spirochetes and rabbit *Treponema pallidum* after similar treatment in our experience and as noted earlier by Turner and Brayton.[19] The fact that Hampp[20] was able to preserve cultures of oral spirochetes by lyophilization, whereas Turner[21] found this somewhat more drastic method unsatisfactory for pathogenic treponemas, again suggests the greater resistance of the oral forms.

Of the routes of inoculation and the animal hosts tested with fusospirochetal exudates, the subcutaneous method in the guinea pig appears to be most useful for maintaining the exudate by passage, and no less satisfactory than the other methods for titration. Relatively large volumes of exudate were most often obtained in the subcutaneously inoculated guinea pig; and, although both hamster and mouse seem slightly more susceptible, the differences are small and do not offset the advantage of a more characteristic and more easily recognized picture of fusospirochetal infection in the guinea pig.

It is apparent that experimental fusospirochetal infection is of low infectivity, since roughly the equivalent of 0.01 ml. of undiluted exudate was required for a minimal response. The apparently greater sensitivity of mouse grain or developing chick embryos seemed to depend on lowered resistance to component members of the fusospirochetal flora, since progressive disease resulted without proliferation of spirochetes. Larger inocula in the adult hosts tended to yield a higher proportion of fatal infections with considerable ir-

regularity. Yet, as much as the equivalent of 0.1 ml. of undiluted exudate did not kill uniformly by the subcutaneous or intraperitoneal routes; and, indeed, 1 of 6 guinea pigs inoculated subcutaneously (Table I) and 1 of 3 guinea pigs and 2 of 5 mice inoculated intraperitoneally (Table II) failed to become infected when given this or a larger dose. These findings are in line with considerable experience in this laboratory with passage of fusospirochetal infection by subcutaneous inoculation of guinea pigs, in which dosages of 0.1 to 0.3 ml. of undiluted exudate have been found most suitable.

The results of intracerebral inoculation of mice and of inoculation of developing chick embryos by various routes suggest that these methods may not be suitable for the study of fusospirochetal infection because of excessive sensitivity of the tissues concerned to microorganisms other than spirochetes in the exudates. The occurrence of the complete flora in mouse brain inoculated with the larger doses may have reflected persistence of the inoculum or the result of an infection that was overwhelming from the outset. Only 2 mice, in which typical infection followed inoculation of higher exudate dilutions, showed what was probably true fusospirochetal infection. The findings in chick embryos seem to conform with those of Goodpasture and Anderson,[22] who showed that several bacterial species usually found to be nonpathogenic for adult animals (*Staphylococcus albus, Streptococcus viridans,* or *Aerobacter aerogenes*) might yield fatal infection in the chick embryo after inoculation on the chorioallantoic membrane. In the present experiments, dissemination of the inoculum through the fluid and tissue of the egg may have permitted fatal infection with the rapidly proliferating cocci and bacilli before the spirochetes had time to multiply. Infection in which spirochetes seemed to participate resulted, without regularity, only when such dissemination was interfered with by mixing the inoculum with agar.

SUMMARY

Fusospirochetal exudates were derived from a case of chronic suppurative periodontoclasia, passed through guinea pigs at least 14 times, and stored when necessary by freezing in CO_2 ice. Such frozen exudates, when thawed rapidly after storage for as long as 13 months, were found to contain typical microorganisms with apparently unimpaired motility, and to yield characteristic infection with dosages similar to those required with fresh exudates.

The following hosts were found susceptible to infection by the routes noted: guinea pig, subcutaneous or intraperitoneal; hamster, subcutaneous; and mouse, intraperitoneal or intracerebral.

Infectivity, as determined by successive dilution, is uniformly low, about the equivalent of 0.01 ml. of undiluted exudate being required for a minimal response. There was considerable variation in individual response, for example, the equivalent of 0.1 or 0.2 ml. of undiluted exudate was frequently a fatal dose,

but in some instances failed to infect. No marked differences in infectivity were correlated with species or route of inoculation among the adult hosts.

Differences in pattern of response among the hosts and routes employed tend to favor subcutaneous inoculation in the guinea pig both for passage and for titration of infectivity.

The intracerebral route in mice, and all routes of inoculation tested in chick embryos, seemed less suitable than other methods, apparently because of excessive sensitivity to infection with microorganisms other than spirochetes. Typical fusospirochetal infection occurred in only 2 of 54 intracerebrally injected mice, and could be obtained irregularly in chick embryos only by injecting agar with the inoculum into the amniotic sac. Such infection in chick embryos could not be passed to fresh embryos.

Mice that survived intracerebral inoculation seemed as susceptible as previously uninoculated mice to subsequent intraperitoneal infection.

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THE ACTION OF FLUORINE UPON THE CALCIFICATION OF THE DENTIN IN RATS WITH LOW CALCIUM RICKETS

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THE effect of sodium fluoride, when injected once, upon the calcification of dentin in normal rats is well known from the work of Schour and Smith,[5] and the time relations and detailed analysis of the observed changes have also been studied (Irving[2]; Irving and Weinmann[4]). The latter authors have pointed out that this histological pattern is found in many instances of experimental disturbances of mineral metabolism. In essence, the predentin existing at the time of the fluorine injection remains uncalcified, or calcifies only slightly, and is often separated from the dentin already calcified by a narrow hypercalcified strip, the calciotraumatic line. The predentin matrix being formed at the time of the injection becomes hypercalcified. The border between hypocalcified and hypercalcified zone has been called the experimental dentin line by Irving and Weinmann.[4] This is the first part of a hypercalcified layer of variable width, which gradually merges with dentin calcified in the usual manner. These phenomena take place at about the same time as the predentin would have normally calcified and are variants of the usual process.

When animals with well-marked high calcium-low phosphorus rickets are injected once with NaF solution, the same changes occur when the predentin calcifies.[2] This is seen best toward the incisal end of the tooth where the predentin is not so wide as it is at the formative end. Even so, owing to the wide predentin and slower calcification rate, the changes caused by fluorine may not appear for some days. The predentin formed at the time of the injection is somehow altered so that when it does ultimately calcify, it does so in an abnormal manner, though the change is not visible histologically as long as it remains uncalcified matrix.

When animals with low calcium-high phosphorus rickets are injected once with NaF solution, a fine hematoxylin staining line is laid down in the predentin forming at the time of the injection (Irving[2]). This is first visible about twelve hours after the injection, and with the lapse of time, it gradually moves across the predentin as fresh matrix is incrementally laid down. Measurements have already shown that this line corresponds to the experimental dentin line of Irving and Weinmann, and in low Ca rickets this line becomes prematurely calcified. No study has been made of the tooth when the predentin containing this line ultimately calcifies. In only four specimens from the writer's existing material has this been seen since, owing to the very wide predentin and very slow calcification rate, it takes up to ten or more days for the affected predentin to calcify. The present paper reports the appearance seen in the four specimens mentioned and also the results of experiments in which the process of calcifica-

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tion was speeded, after the NaF had been given, by a small dose of vitamin D. The picture seen was the same in both groups, so that vitamin D had not altered the response of calcifying dentin.

EXPERIMENTAL

Young rats of the Wistar Institute strain were used. When they had attained a weight of between 50 and 60 grams, they were placed on the Diet 3 previously used (Irving[2]). This diet was based on the Steenbock and Black rachitogenic diet, but modified so that the Ca content was 0.12 per cent and the P was 0.42 per cent, the Ca:P ratio being 0.29. After 28 days on this diet the rats were treated as follows:

Group I consisted of 4 rats. They were injected subcutaneously once with a 2 per cent NaF solution at a dose level of approximately 8 mg. F per kg. body weight. After a further 10 days on Diet 3 they were killed and the dental tissues examined.

Group II consisted of 20 rats. Fifteen of these were injected in the same way as those of Group I. One day later they were given one dose of 27 I.U. of vitamin D in arachis oil by mouth. They were then continued on Diet 3 and were killed as detailed in Table I. One control rat was examined after 28 days on Diet 3, and 4 others were killed 4, 6, 8, and 8 days respectively after vitamin D dosage only. The complete timetable is given in Table I.

TABLE I
RATS OF GROUP II

RAT NUMBER	DAYS OF SURVIVAL AFTER:	
	NaF DOSAGE	VITAMIN D DOSAGE
1061, 1164, 1165	3	2
1062, 1071	5	4
1166, 1167	6	5
1063, 1073	7	6
1064, 1168, 1169, 1076	9	8
1170, 1171	12	11
1070	–	4
1072	–	6
1074, 1075	–	8
1163	Killed after 28 days on Diet 3	

Group III consisted of 6 rats. These were injected with NaF solution in the same way as those of Group I, and at the same time were given 9.2 I.U. of vitamin D in arachis oil in one dose by mouth. After a further 10 days on Diet 3, they were killed and the dental tissues examined.

Histological Examination.—The skulls were fixed in formol-saline and decalcified in nitric acid-formol saline. The lower jaws were removed, and after embedding in paraffin, the upper incisor teeth were cut semiserially in longitudinal section. They were stained with hematoxylin and eosin, and in the case of Group II rats, also by the method of silver impregnation (Gömöri[1]). The changes in the labial dentin and predentin, where they are most clearly seen, were examined. Those on the lingual side also were noted.

RESULTS

Group I.—By 10 days after the NaF injection, the calcified experimental dentin line and the slow calcification of the dentin had met and blended at the formative end of the tooth (Fig. 1). When this happened the line was still conspicuous as a thin hypercalcified layer on a background of dentin. The predentin had calcified right up to this line on the enamel side, but on the pulpal side of the line a narrow uncalcified or hypocalcified zone was to be seen. The predentin calcified on the pulpal side of this hypocalcified zone and appeared the same in density of calcification as that on the enamel side of the line. More incisally, the dentin had calcified in an interglobular fashion. Here the experimental dentin line was not so conspicuous, but the hypocalcified layer was clearly seen (Fig. 2).

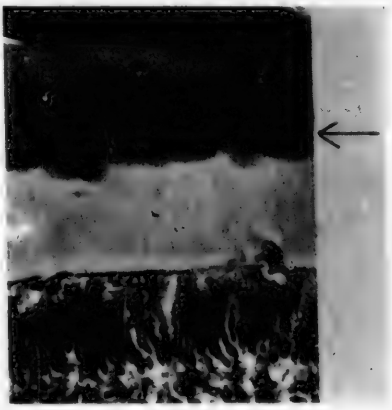

Fig. 1.

The microphotographs are of the labial incisal dentin and predentin in formative or mid-region of tooth. All animals had been on low Ca rachitogenic diet for 28 days before experimental procedures described. Sections in Figs. 1 to 4 were stained with hematoxylin and eosin, those of Figs. 6 to 8 by silver impregnation. Orig. mag. ×270.

Fig. 1.—Rat 135, injected once with NaF 10 days before being killed. Arrow points to experimental dentin line which has been incorporated into slowly calcifying dentin. On pulpal side of it a narrow hypocalcified zone is seen.

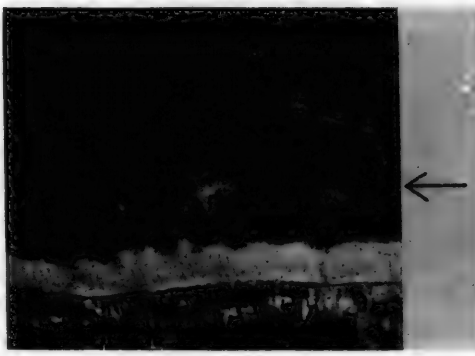

Fig. 2.

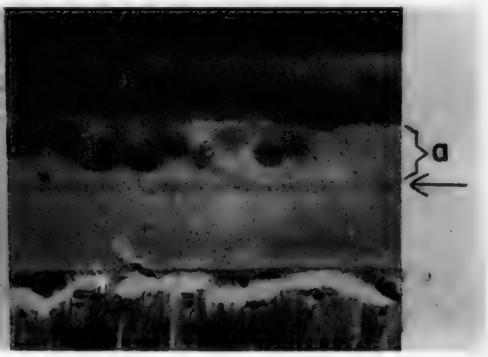

Fig. 3.

Fig. 2.—Rat 135. This part of section is more incisal than that in Fig. 1, and spontaneous calcification is interglobular in nature. Arrow points to site of experimental dentin line which is bounded pulpally by an uncalcified zone.

Fig. 3.—Rat 1062, which was killed 5 days after NaF injection and 4 days after vitamin D dosage. Arrow points to experimental dentin line. Zone *a* contains calcospherites laid down as result of vitamin D action.

Group II.—In this group it was possible to study in stages the process which was complete in the rats of Group I. The effect of vitamin D upon the teeth of rats with low Ca rickets is to cause a calcospherite response which starts near the already calcified dentin and spreads pulpally across the predentin.[3] This gradually consolidates into a mass of interglobular dentin. F causes the appearance of the prematurely calcified experimental dentin line already mentioned. In the early stages after administration of NaF followed by vitamin D, both the calcified line and the calcospherite response could be seen in separate parts of the predentin (Fig. 3). About 6 days after vitamin D dosage, the calcospherites first met and blended with the experimental dentin line. They seemed to pause, their inner edges not extending over this line. By the eighth day, fresh calcospherites were visible on the pulpal side of the line, separated from it by a narrow uncalcified layer (Fig. 4). Thereafter the reaction to vitamin D spread and the calcospherites gradually fused till finally they halted

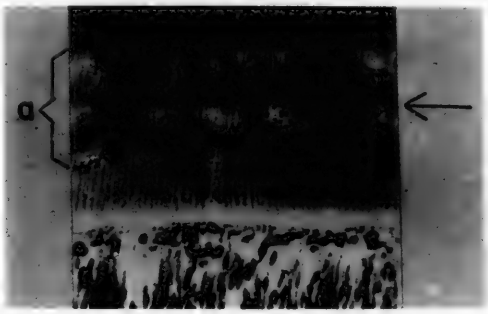

Fig. 4.—Rat 1064, killed 9 days after NaF injection and 8 days after vitamin D dosage. Arrow points to site of experimental dentin line. Calcospherites in zone *a* were laid down as result of vitamin D action. They blended with line on enamel side but left an uncalcified layer on pulpal side. Appearance is same as that in Fig. 2.

at about the normal predentin width from the odontoblasts. A hypo- or uncalcified zone was left however on the pulpal side of the experimental dentin line which persisted no matter how intense the calcification of the dentin ulti-

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Fig. 5.—Semidiagrammatic camera lucida drawings to illustrate changes in labial dentin and predentin of rat's incisor tooth after dosage with NaF and vitamin D. In all cases, 27 I. U. of vitamin was given by mouth one day after injection of NaF.

a = pre-experimental dentin. *b* = pre-experimental dentin or predentin. *c* = post-experimental dentin or predentin. *d* = odontoblasts. Arrow points to experimental dentin line or to site of this line.

Some calcification may have taken place in the pre-experimental dentin *a* as experiment proceeded and thus junction of *a* and *b* is approximate only in later drawings.

A.—Rat 1164, 3 days after NaF injection. Experimental dentin line is in uncalcified predentin, vitamin D reaction having hardly started.

B.—Rat 1071, 5 days after NaF injection. Vitamin D reaction has now begun, but has not met experimental dentin line.

C.—Rat 1063, 7 days after NaF injection. Vitamin D reaction is now more marked and has met experimental dentin line Calcospherites seem to have fused along this line and a few have developed on pulpal side of it.

D.—Rat 1168, 9 days after NaF injection. Large calcospherites have now developed on pulpal side of experimental dentin line, leaving an uncalcified space.

E.—Rat 1170, 12 days after NaF injection. Vitamin D reaction is now quite intense. Experimental dentin line has been incorporated into new calcification and is present at level marked by arrow. Thin uncalcified layer is conspicuous against background of calcified dentin.

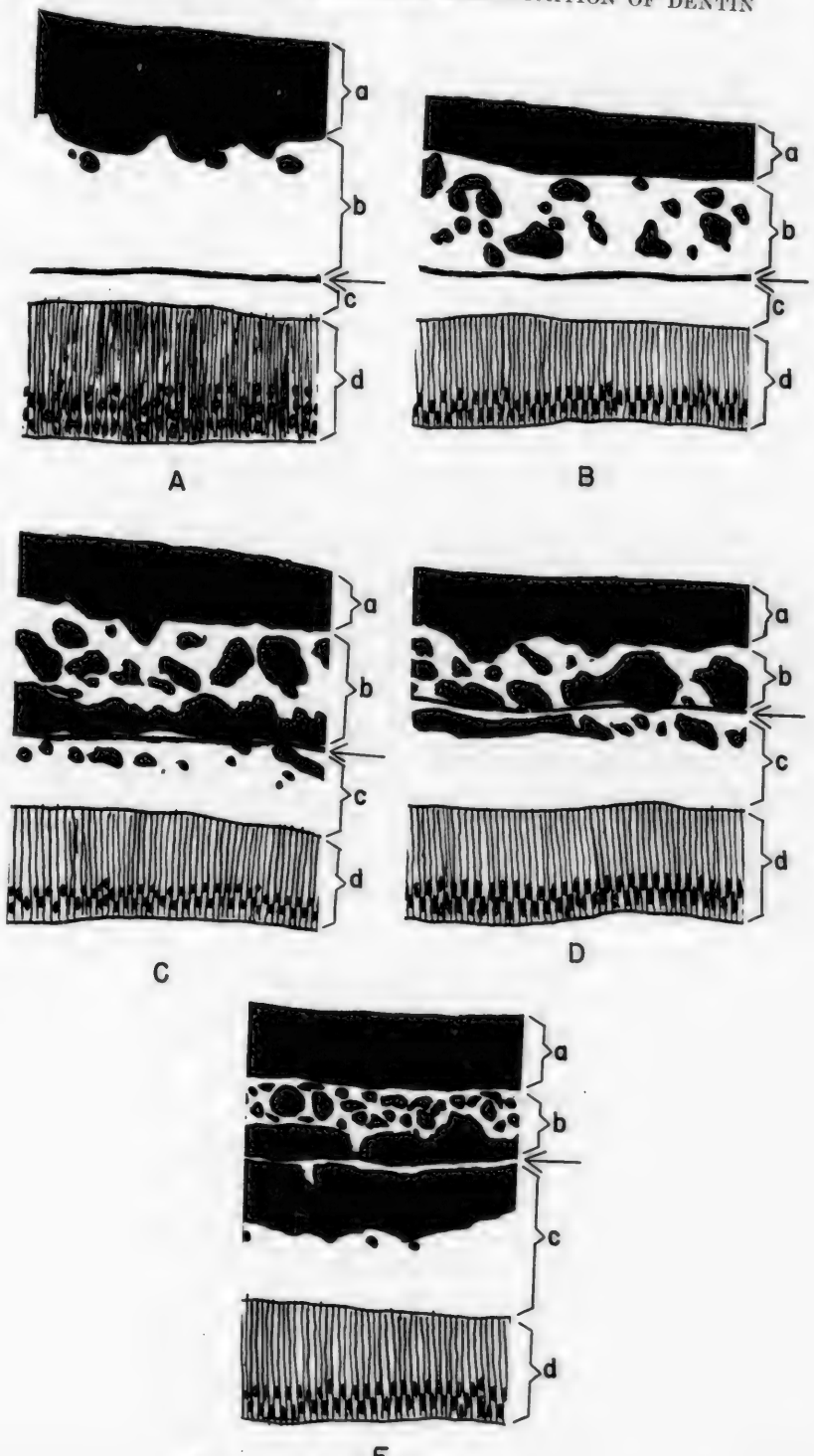

Fig. 5.—*(See opposite page for legend.)*

mately became. The process is illustrated diagrammatically in Fig. 5. The F reaction with the newly calcified dentin was first seen toward the formative end of the tooth. As calcification proceeded, it was visible farther and farther toward the incisal end. It will be noted by comparing Figs. 2 and 4 that the same reaction was obtained in the animals of this group as was seen in those in which calcification occurred spontaneously.

The picture described above was the one seen with the hematoxylin and eosin stain. Silver staining confirmed this histological appearance. With silver impregnation, the experimental dentin line was visible at all stages of the experiment as a fine intensely argyrophobic line (Figs. 6, 7, 8). Irving and Weinmann[4] have shown that in calcified dentin, the cementing substance which holds the calcium salts stains well with hematoxylin and poorly with silver. The silver reaction of the line is thus due to a relative decrease in the dentin fibrils, which stain well with silver, and an increase in the interfibrillar cementing substance,

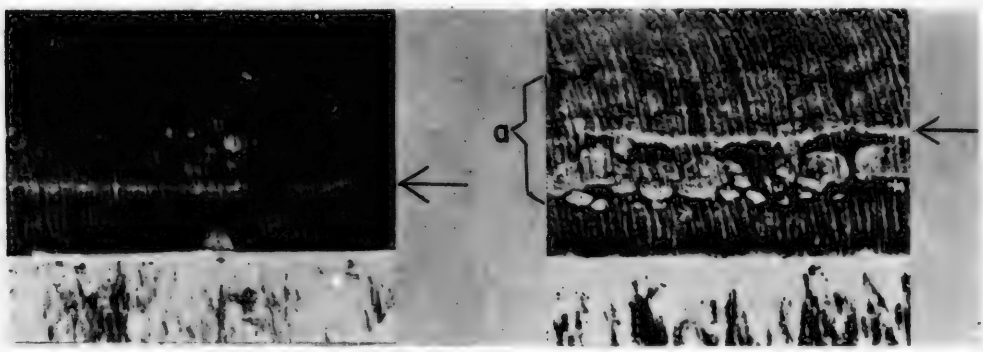

Fig. 6. Fig. 7.

Fig. 6.—Rat 1062. Experimental dentin line is seen as strongly argyrophobic layer. Calcospherites were laid down as result of vitamin D action.

Fig. 7.—Rat 1064. Arrow points to experimental dentin line. Calcospherites in zone *a* were laid down as result of vitamin D action. Note that they blended with line on enamel side, but left an argyrophilic layer on pulpal side of it. Argyrophobic nature of calcospherites is quite marked.

and thus in calcium salts, per unit of cubic volume of dentin. In the early stages, the experimental dentin line was visible as an argyrophobic line in the predentin; the calcospherites, caused by vitamin D being hypercalcified, also took up the silver stain poorly (Fig. 6), but were not as argyrophobic as the line. In the later stages the experimental dentin line stood out very sharply, hardly staining at all with silver (Figs. 7 and 8). On the enamel side, calcospherites could be seen laid down up to and blending with the line. On the immediate pulpal side of it, an irregular argyrophilic layer was seen. This had the same staining reactions as predentin, and could be regarded essentially as uncalcified matrix. On the pulpal side of this, a layer of argyrophobic calcospherites were laid down owing to the action of vitamin D.

Group III.—The same picture was visible as was present in the rats of Group II which were killed late in the experiment.

DISCUSSION

An identical reaction was obtained in the dentin after NaF injection both in teeth which were calcifying spontaneously and also in those in which the process was accelerated by vitamin D. This shows that the vitamin merely stimulated a process which was occurring in any case and that deductions drawn from the vitamin-dosed rats can be applied to those which were not so treated.

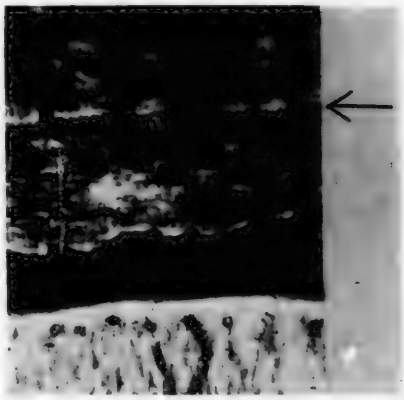

Fig. 8.—Rat 1171, killed 12 days after NaF injection and 11 days after vitamin D dosage. Arrow points to experimental dentin line. Appearance is similar to that of Fig. 7, but calcification caused by vitamin D is more advanced.

The reaction caused by NaF is in essentials as follows:

A. A prematurely calcifying experimental dentin line appears in the predentin.

B. When spontaneous calcification takes place, this line blends with dentin on its enamel side, but is separated from that on the pulpal side by a narrow uncalcified layer.

Both reactions are quite different from what is seen following F injection in normal animals or those with high Ca rickets. At first sight it might appear that this interpretation is wrong, that the line is really the calciotraumatic line, since it is followed by a hypocalcified layer. Careful measurements,[2] which have been repeated, have however shown that this line undoubtedly corresponds to the experimental dentin line. Furthermore, in normal teeth, the hypocalcified layer is predentin existing at the time of the injection. The same is found in high Ca rickets; here the hypocalcified layer is much wider because of the greater width of the pre-existing predentin. No such zone was found in the present results. The predentin was excessively wide; and had this type of hypocalcified zone appeared, it would have been most conspicuous. The results with

the silver stain confirmed those with hematoxylin and eosin and supported the interpretation given previously.

It must therefore be concluded that in low Ca rickets F has a special effect upon calcifying dentin. The predentin existing at the time of the injection ultimately becomes completely calcified; the hypercalcified layer is of very short duration in time and is succeeded by a narrow uncalcified zone. The reason for the sequence of events in normal teeth after F injections has not yet been found, and the same is true for the present findings. As with the response in normal teeth, the peculiarity which caused the response to F must have been inherent in the matrix as laid down, but with low Ca rickets part of this response became demonstrable with staining methods almost immediately after the matrix had formed.

In the Group III animals, the same response was seen even when NaF and vitamin D were given simultaneously. NaF probably acts almost immediately on the odontoblasts and its action is relatively short lived, whereas vitamin D, given by mouth, takes at least 30 hours before it begins to act at the dose level used.[3] The premature calcification of the line is not interfered with provided F has acted before the action of vitamin D begins. If vitamin D acts first, then the premature calcification is suppressed, as is described in a subsequent paper.

SUMMARY

1. This paper reports the effect of one injection of NaF solution upon dentin calcification in low Ca-high P rickets.

2. Animals were studied (1) in which spontaneous calcification had occurred, and (2) in which calcification had been accelerated by a simultaneous or subsequent dose of vitamin D.

3. As already reported, fluorine causes the formation of a fine calcified line, almost immediately stainable by histological methods, in the predentin forming at the time of the injection. This is the same as the experimental dentin line in normal teeth.

4. In both groups of animals mentioned in (2) above, when the predentin affected by the injected F calcified, calcification on the enamel side of the experimental dentin line occurred up to and blended with this line. On the pulpal side of the line, a fine irregular zone of matrix did not calcify at all. Subsequently formed matrix calcified in the usual way.

5. This reaction is quite different from that which occurs after F administration in normal animals or those with high Ca rickets.

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THE IN VITRO PRODUCTION OF A "YELLOW-BROWN MELANIN-LIKE PIGMENT" IN THE ORGANIC MATRIX OF NONCARIOUS HUMAN TOOTH CROWNS BY METHYLGLYOXAL (PYRUVIC ALDEHYDE) AND ACETOL (ACETYL CARBINOL)

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IN a symposium on the evaluation of dental caries control technics held at Ann Arbor, Michigan, in September, 1947, the nature and significance of the yellow-brown stain in carious lesions of the enamel and dentin aroused much interest and discussion.[1] Previously Gottlieb[2] had incorporated this manifestation into a theory of tooth decay which challenged the hitherto widely accepted acid decalcification concept of the mechanism of dental caries. According to Gottlieb, dental caries is essentially a proteolytic process which proceeds along the organic pathways of the enamel and dentin through the media of the lamellae or the uncalcified prism sheaths. The process is associated with the development of a yellow-brown pigment which appears from the time the tooth structure is first invaded. Gottlieb postulated that the pigment is produced by microorganisms present in the carious lesion. Neither he nor anyone else, however, has been able to identify the causative organism.

As early as 1930, Bibby[3] suggested that the pigmentation orginated from the products of decay per se, and that it might be the result of caries activity. Deakins[4] hydrolyzed the organic matrices of carious human teeth in 20 per cent HCl at 110° C. for 24 hours and obtained a black insoluble pigment which resembled melanin in its chemical composition. No such pigment was recovered from similarly treated noncarious tooth matrices. He did note, however, a soluble coloring matter which may have been derived from exogenous colored substances (foodstuffs, tobacco, dentifrices, etc.) deposited either upon or within the surface of the enamel and exposed dentin. This was obtained from both the carious and unaffected dentinal matrices.

The role of acids produced during the bacterial degradation of glucose in the initiation of the carious lesion has been vividly demonstrated by Dietz.[5] The degradation of glucose by microorganisms has been found to be a complex process characterized by the formation of many intermediate compounds, some of which contain carbonyl groups. It is of interest that a number of carbonyl-containing compounds may react with the amino derivatives of proteins to form melanoidins. Zerban[6] suggested three such compounds, namely, methylglyoxal (pyruvic alde-

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hyde), acetol (acetyl carbinol), and hydroxymethylfurfural. Methylglyoxal and acetol have been demonstrated to be degradation products of glucose.[7, 8] The former is believed by many students of fermentation to be the primary product formed in the dissimilation of glucose.[8]

These findings suggested to us that the insoluble yellow-brown pigment observed in carious lesions might be produced by the interaction of the carbonyl-containing degradation products and the organic element of the tooth. The present study was undertaken to test this hypothesis by treating the organic matrices of noncarious tooth crowns with methylglyoxal and acetol in the absence of proteolytic organisms and exogenous sources of pigmentation in an effort to obtain an insoluble yellow-brown pigment.

MATERIALS AND METHODS

Freshly extracted caries-free teeth of human beings were scrubbed with a brush and pumice and washed with distilled water to remove any food debris and clinging organic matter. They were placed in a solution containing 4 per cent formaldehyde in 50 per cent ethyl alcohol and stored in a refrigerator. Six teeth treated in this manner were covered in their entirety with pink base-plate wax except for a small window approximately 1×0.5 cm. which was left exposed on the crown. The teeth were placed in 25 c.c. 0.1 N lactic acid for 72 hours. The solution was changed at the end of each 24 hours. At the end of the 72-hour period, they were removed, rinsed in distilled water, and divided into three pairs. The pink base-plate wax was stripped from one tooth in each pair to rule out the possibility of constituents of the wax entering into the formation of the pigment. The first pair, which was to serve as the controls, was immersed in distilled water, the second in 5 c.c. of a 30.4 per cent solution of methylglyoxal, and the third in 2 c.c. of acetol. The flasks containing the three pairs of teeth were kept at 37° C.

Three additional freshly extracted caries-free human teeth, cleaned and stored in the manner described above, were severed at the cervical region. The pulps were removed and the crowns were decalcified overnight in 25 c.c. of 5 per cent HCl. They were then washed in distilled water until free from acid. One served as the control and was left in distilled water, the second was placed in a flask containing methylglyoxal, and the third was immersed in acetol. At the end of 72 hours, the crowns were quartered and tested for an insoluble black pigment by the method described by Deakins.[4] The sections were dried in vacuo at 110° C. for 2 hours and kept in a $CaCl_2$ desiccator until hydrolyzed in 1 c.c. of 20 per cent HCl at 110° C. for 24 hours. The residues were washed with distilled water and filtered through glass wool by suction.

OBSERVATIONS

Decalcified areas were observed in each of the six teeth exposed to the action of 0.1 N lactic acid for 72 hours. These areas followed the outlines of the windows cut in the wax. Coronal dentin was exposed in each instance, the color varying from a light gray to a faint yellow-white.

At the end of the 72-hour immersion in acetol the color of the exposed dentin (and cementum of the tooth from which the base-plate wax had been stripped away) ranged in color from yellow to yellow-brown. The color in the dentin was much deeper in hue than that in the enamel and cementum. The pigment was insoluble in water and followed the path of the dentinal tubules. The color change was noted within 6 hours after the tooth was exposed to the action of acetol.

The coronal dentin (and cementum of the tooth from which the wax had been stripped away) developed a color ranging from yellow-brown to brown following immersion in methylglyoxal for 72 hours. The color in the dentin was much deeper in hue than that in the enamel and cementum. In cross section the pigment was seen, with the aid of a hand lens, to follow the course of the dentinal tubules. It was insoluble in water, and was noted within 8 hours. The control teeth, kept in distilled water, did not undergo a color change.

The decalcified crowns exposed to methylglyoxal (Fig. 1) and acetol were yellow-brown and yellow-orange in color respectively at the end of 72 hours. These crowns, when quartered and tested by Deakins' method, yielded black insoluble pigment granules. The crown which served as the control did not undergo a color change and did not contain the insoluble black pigment granules (Fig. 2). In each instance some soluble coloring matter was recovered, however, in the filtrate following suction.

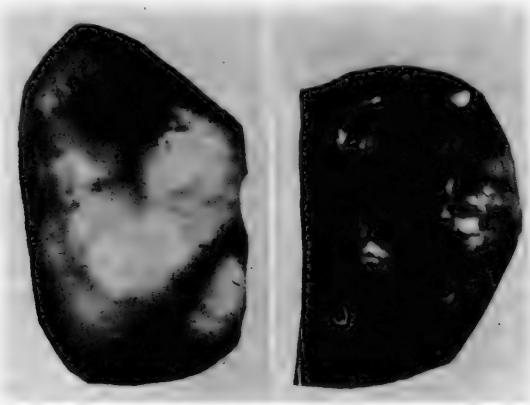

Fig. 1.—Decalcified sections of crowns of noncarious human teeth. The section on right was immersed in methylglyoxal for 72 hours, and developed deep yellow-brown color. The section on left served as control and was immersed in distilled water. It did not undergo color change.

DISCUSSION

While little is known of the source or origin of the naturally occurring yellow-brown pigment observed in carious teeth, it has been assumed to be melanin because of its color, relative insolubility in saliva, apparent formation in situ, and chemical composition.[4] There appears also to be general agreement that the organic element of the enamel and dentin is implicated in some way.[2, 9, 10]

The findings reported in this study show that certain carbonyl-containing products of carbohydrate degradation are capable of reacting with the organic elements of the enamel and dentin to form insoluble pigments which range in color from yellow to brown when seen in situ. The pigment thus produced was similar in appearance to that found by Deakins in carious teeth. Neither proteolytic bacteria nor exogenous sources of coloring matter were required to achieve these results. It would seem, therefore, that the presence of a yellow-brown pigmentation in decayed teeth can be reconciled with Miller's acid decalcification theory of the mechanism of dental caries, in which he states that "Dental decay is a chemico-parasitical process consisting of two distinctly marked stages: decalcification, or softening of the tissue, and dissolution of the softened residue. In the case of enamel, however, the second stage is practically wanting, the decalcification of the enamel practically signifying its total destruction."[11]

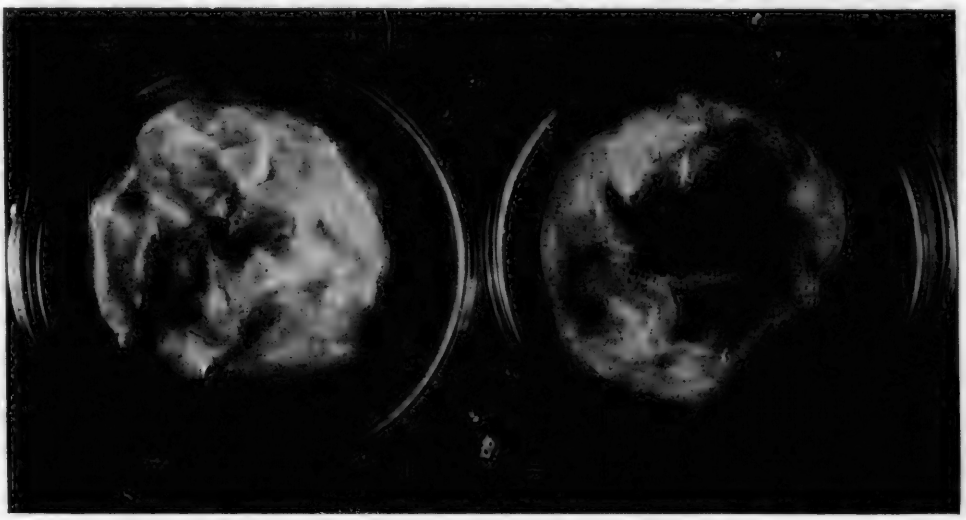

Fig. 2.—The black insoluble pigment granules in dish on right were recovered from quarter-section of decalcified crown exposed to action of methylglyoxal. As may be seen in dish on left, quarter-section of decalcified crown which served as control, when treated by Deakins' method, did not yield any black insoluble pigment granules.

SUMMARY AND CONCLUSIONS

1. When decalcified noncarious tooth crowns and parts of crowns were exposed to the action of either methylglyoxal or acetol for 72 hours, a pigmentation varying in color from yellow to brown was produced which followed the organic pathways of the tooth, and appeared to be melanin-like in nature.

2. The pigment, formed by the interaction of the organic elements of the enamel and dentin with two of the carbonyl-containing products of carbohydrate degradation, appears to be identical with that found in the naturally occurring carious lesions.

3. These findings lend support to the acid decalcification theory of the mechanism of dental caries.

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REPLICA STUDIES OF CHANGES IN TOOTH SURFACES WITH AGE

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A METHOD for the study of the smooth surfaces of teeth by means of optical microscopy of metal-shadowed collodion replicas has been described in previous publications.[1, 2] In these earlier studies it was found that the details visible on such replicas include enamel rod ends, perikymata, scratches, cracks, and representations of various developmental and acquired defects. Observation of several thousand replicas taken from surfaces of extracted teeth selected at random demonstrated clearly that classification of surfaces according to the type and relative amount of structural detail visualized would not only be difficult, but also might be inaccurate. For the present study replicas were taken from the surfaces of teeth classified according to the age of the individuals from whom the teeth were extracted. It became evident that the surfaces of unerupted and recently erupted teeth closely resemble one another, but differ greatly from those of older teeth which have been exposed to attritional and other environmental influences. It also became evident that alterations in surface structure occur with a regularity which definitely can be related to age. For these reasons the unerupted or recently erupted tooth surface has been chosen as the standard of comparison for details visible on tooth surfaces. In the present paper the character of such surfaces will be described first, and then the appearance of surfaces on teeth from older individuals will be described.

Table I is a summary, according to age groups, of the number of individuals, teeth collected, and replicas taken. Approximately half of the teeth studied were carious, and slightly over half of the surfaces studied were on carious teeth. About 80 per cent of the surfaces on carious teeth were noncarious and 20 per cent, carious. Since it was not possible to discern any structural differences between surfaces of noncarious teeth, noncarious surfaces of carious teeth, and the intact portions of carious surfaces, all surfaces have been considered together in the tables which follow.

Immediately after extraction the teeth were placed in 5 per cent formalin. The surfaces were cleaned prior to preparation of the replicas by brushing with a rotating dental prophylaxis brush and a water-abrasive mixture containing Linde Abrasive Powder A, which has a maximum particle size of 0.3 μ. It was found in preliminary tests that the surfaces were cleaned efficiently and without appreciable alteration of structure by this abrasive. Although a few short, shallow scratches were introduced, they were negligible compared to the many long, deep scratches that resulted from the use of pumice or coarse alumina.

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TABLE I
SUMMARY OF REPLICAS TAKEN

AGE	NO. INDIVIDUALS	TOTAL NO. TEETH	TOTAL NO. SURFACES STUDIED						TYPE SURFACE STUDIED			NO. CARIOUS TEETH	NO. NON-CARIOUS SURFACES ON CARIOUS TEETH	NO. CARIOUS SURFACES ON CARIOUS TEETH	TOTAL NO. SURFACES ON CARIOUS TEETH
			INCISORS	CANINES	PREMOLARS	MOLARS	UNERUPTED MOLARS	TOTAL	BUCCAL LABIAL	LINGUAL	PROXIMAL				
10 to 14	42	93	7	0	145	31	—	183	66	56	61	16	37	7	44
15 to 19	40	69	11	2	59	46	32	150	50	36	64	33	45	19	64
20 to 29	74	140	40	7	57	88	51	243	78	64	101	60	97	34	131
30 to 39	57	100	26	10	46	97	9	188	70	57	61	75	123	20	143
40 to 49	32	100	78	48	33	53	—	212	83	53	76	73	135	36	171
Over 50	42	137	110	47	44	58	4	263	118	61	84	77	115	33	148
Total	287	639	272	114	384	373	96	1239	465	327	447	334	552	149	701

The surfaces were examined prior to preparation of the replicas with a 2.5 power binocular loupe, and the presence or absence of caries and visible structural detail was recorded.

Collodion replicas were prepared according to the technic previously described[1] and were shadowed at right angles to the cemento-enamel junction by evaporation of 65 mg. of silver from a tungsten filament located 6 cm. behind and 3 cm. above the replicas. The shadows thus produced were roughly twice the length of the objects responsible for them. In studying the accompanying micrographs it is necessary to remember that in the replicas elevations are produced by depressions in the tooth surface and vice versa.

The replicas were examined with an optical microscope at magnifications of 100×, 430×, and 950×. They were not grouped according to age in advance, but were examined at random and without information regarding the history of the teeth. Tabulations according to age were made subsequent to the completion of the microscopy.

SURFACE STRUCTURE OF UNERUPTED AND RECENTLY ERUPTED TEETH

The three principal structures visible on the replicas are perikymata, enamel rod ends, and cracks. Perikymata are seen over the entire areas of all surfaces. Though perikymata have been described as external manifestations of the ends of the striae of Retzius,[3, 4, 5] their origin and development and their relation to the underlying enamel are not yet understood. The general form of their pattern is the same on all surfaces, but they demonstrate marked differences in width, depth, distance apart, and course on individual surfaces and on different teeth. (Figs. 1, 2, 3, 4, 7, 11, 13, 14.) Measurements on 100 replicas of the number of perikymata and their distance apart were made with the aid of a micrometer disc. The resulting data of Table II show that there is a regular decrease in the concentration of perikymata from the cemento-enamel junction (Figs. 1 and 14) to the occlusal or incisal edge of the surface (Figs. 11 and 13). The greatest variation in number (Table II, Column 3) occurs in the immediate region of the cemento-enamel junction. Here the perikymata are often so close together that it is difficult to count them.

TABLE II
DISTRIBUTION OF PERIKYMATA

REGION IN MM. FROM CEJ*	AVERAGE NO. PERIKYMATA	VARIATION IN NO. OF PERIKYMATA	AVERAGE DISTANCE APART (μ)
0-0.7	22	12-50	32
0.7-1.4	18	12-28	39
1.4-2.1	14	8-26	50
2.1-2.8	11	5-28	64
2.8-3.5	9	6-22	78
3.5-4.2	8	5-15	88
4.2-4.9	7	5-15	100
4.9-5.6	7	6-10	100
5.6-6.2	7	6- 9	100
6.2-6.9	7	6- 9	100

*CEJ means cemento-enamel junction.

Perikymata appear to be continuous around the tooth, as was demonstrable on replicas in which the collodion covered parts of adjacent surfaces. In the case of teeth in which the enamel on one surface extends further toward the apex of the root than on another, the number of perikymata near the cemento-enamel junction of the shorter surface is similar to that in the region immediately adjacent to it on the longer surface. Accordingly, at the cemento-enamel junctions perikymata are usually closer together on the longer surface than on the shorter.

The course of most perikymata is sinuous and generally regular across the tooth surface. The greatest irregularities occur in the first millimeter above the cemento-enamel junction and are most often found on the surfaces of molars.

The perikymata on the cervical third of a surface appear to be inclined toward the cemento-enamel junction (Fig. 1). In order to demonstrate this inclination, specimens have been shadowed in two ways: (a) with the replica placed so that the occlusal or incisal edge was nearest to the source of the metal, and (b) with the replica rotated through 180° so that the cemento-enamel junction was nearest the source. When shadowed from the former direction the elevated ridges on the replicas representing the perikymata cast definite shadows onto immediately adjacent regions (Fig. 3). When the ridges were shadowed from the latter direction such shadows were not cast, but there was an accumulation of metal against the near sides of the ridges, and a fairly even coating on the far inclines (Fig. 4). This indicates that these perikymata have a long, gently sloping wall and a short steep wall, the latter being nearer to the cemento-enamel junction. On the middle third of a surface, the walls of perikymata form more or less symmetrical V's; near the occlusal or incisal edge, the sharp angulation disappears (Fig. 11). Fig. 5 is a schematic drawing of the profile of a surface which could produce replicas similar to those actually obtained, and a representation of the course taken by perikymata across a typical surface. It has been stated[3] that the perikymata represent an overlapping of sheets of enamel, but the available evidence does not prove or disprove this hypothesis.

Nearly all surfaces of unerupted or young teeth are completely covered with a pattern of concavities which correspond to structures earlier described as ends of enamel rods.[3, 5, 6] These concavities appear as minute elevations on the replicas and are approximately 6 μ in diameter (Figs. 1, 2, 6, 7). There is considerable variation in their depths, both on the same surface and on different surfaces. The shallow depressions of Figs. 6 and 7 may be found anywhere on a surface; relatively deep concavities, such as those of Fig. 2, are seldom seen on cervical regions.

About 90 per cent of the surfaces examined have shown fairly evenly spaced structures, resembling cracks, which originate at the cemento-enamel junction (Fig. 7). There is considerable variation in the number of these cracks but no visible difference in their appearance on various surfaces and on different types of teeth. The great majority are from 100 to 1,000 μ in length, but a few traverse the entire surface and reach the occlusal or incisal edge. Long cracks appear to be slightly wider than short ones. It is not known whether the cracks

Fig. 1.

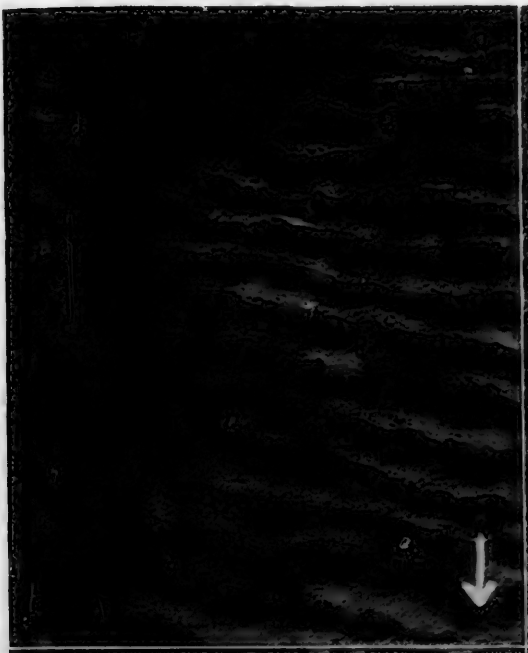
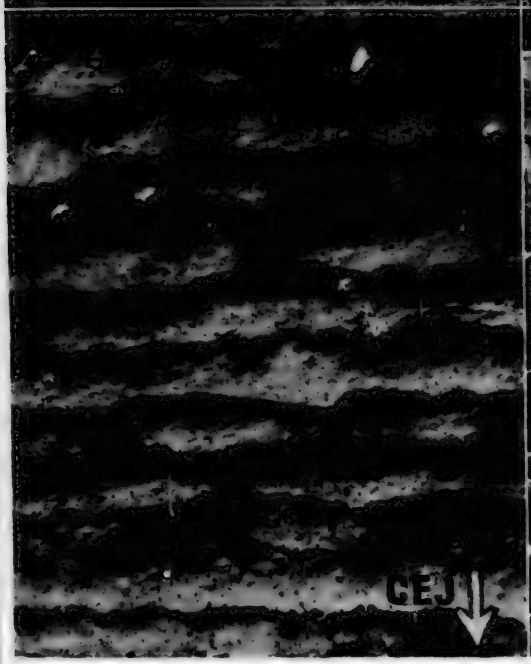


Fig. 2.



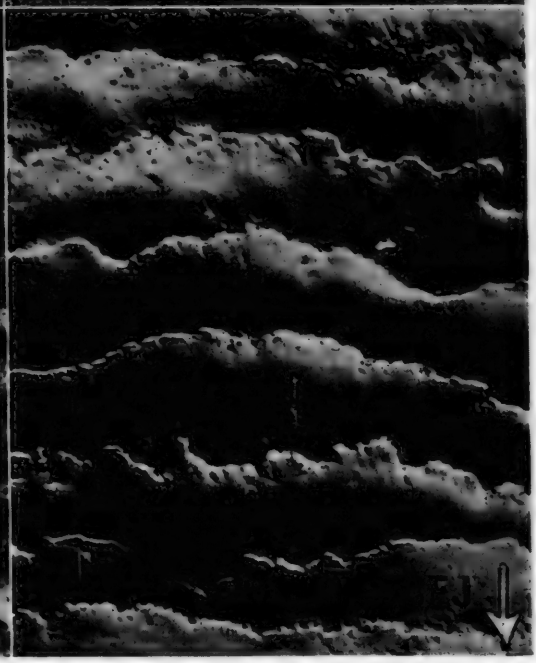


Fig. 3.

Fig. 4.

In the illustrations of this paper, CEJ refers to cemento-enamel junction, and the arrows show its position in relation to the region of the surface shown in the photograph.

Fig. 1.—*Pronounced* structure on the surface of a recently erupted tooth (first premolar, distal surface; age 12). (130×)

Fig. 2.—*Pronounced* structure on the surface of a recently erupted tooth (first premolar, buccal surface; age 14). (130×)

Fig. 3.—Cervical perikymata shadowed from the direction of the occlusal edge. (205×)

Fig. 4.—Cervical perikymata shadowed from the direction of the cemento-enamel junction. (250×)

are formed during tooth development, or are a result of influences acting subsequent to calcification, but the regular arrangement of the short cracks and their presence on unerupted teeth (Table III) suggest that they are not due to damage in handling. No structures have been observed on replicas which can be identified as the outer ends of lamellae. If lamellae exist as sheets of tissue running parallel to the long axis of the tooth, and at right angles to the dentin as some authors have suggested,[7, 8] they could manifest themselves as these cracklike structures. The data of this paper, however, do not show whether or not the observed cracks should be interpreted in this fashion.

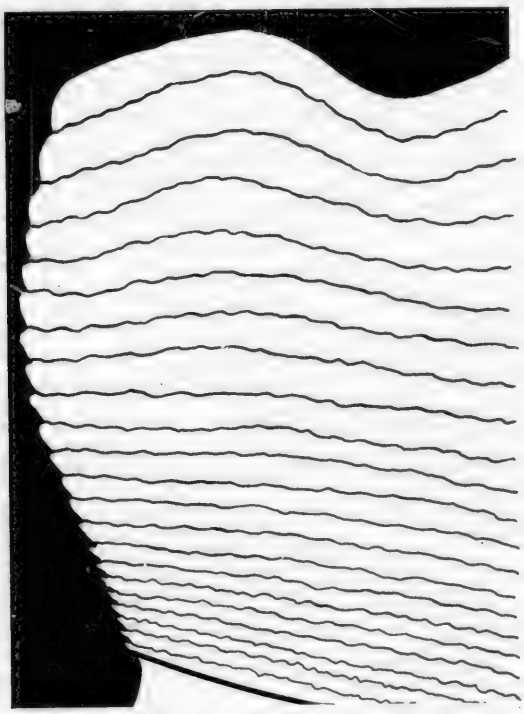


Fig. 5.—Schematic drawing showing profile and course of perikymata.

Experiments were performed to determine the relation between enamel cuticle* and the structures visualized on surface replicas. In order to obtain cuticle for microscopy, use was made of 25 unerupted and 25 recently erupted teeth, the surfaces of which yielded replicas showing pronounced and complete patterns of perikymata and rod ends. These teeth, after being stained with hematoxylin and eosin, were placed in alcoholic nitric acid (5 per cent HNO_3 in 50 per cent C_2H_5OH)[9] until the cuticles became detached. In all cases this cuticle could be observed over the entire surface areas. Though the acid removed most of the stain, enough remained to facilitate observation of the membrane. Pieces of cuticle were placed on slides and shadowed by the same technic

*The term enamel cuticle refers to membrane or membranes covering the enamel surface.

used for the replicas. There was a close resemblance between the structures observed on cuticle treated in this way (Fig. 8) and on surface replicas taken before its removal. Perikymata and rod ends were both discernible, but they were not as pronounced as on the replicas. Apparently there was also some action of the acid alcohol upon the cuticle which showed itself as a generalized microscopic pitting, or roughness, not seen on the replicas.

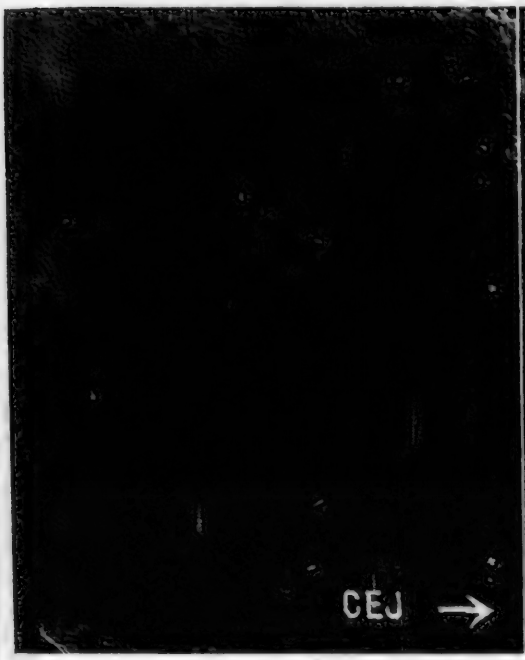


Fig. 6.

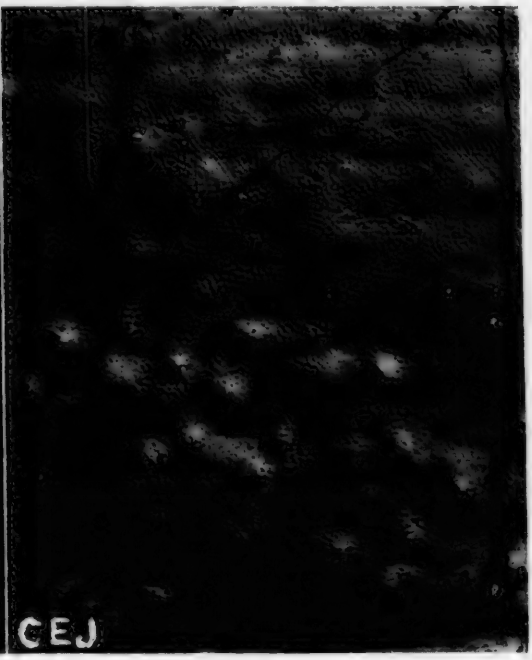


Fig. 7.

Fig. 6.—*Pronounced* structure on the surface of a recently erupted tooth (first premolar, distal surface; age 12). (130×)

Fig. 7.—*Pronounced* structure on the surface of a recently erupted tooth (first premolar, distal surface; age 13). (130×)

Attempts next were made to demonstrate perikymata and rod ends on tooth surfaces freed from cuticle. Replicas of surfaces taken subsequent to removal of cuticle by acid show such deeply etched enamel rods that they cannot be accurate representations of the region immediately beneath the cuticle. Seeking to observe less damaged surfaces, teeth, in groups of five each, were treated with sodium sulfide, urea, or trypsin in various concentrations and for various lengths of time. Cuticle was not dissolved by such means since it could subsequently be floated off with acid. Changes in structure could not be observed on replicas taker before or after treatment with any of these nonacid reagents. It has thus not yet been possible to establish in this direct fashion whether the structures observed on replicas of unerupted or recently erupted tooth surfaces represent enamel surface structure to which a thin membrane has been closely adapted or detail only to be found in the membrane itself.

TABLE III

OCCURRENCE OF STRUCTURES ON TEETH FROM INDIVIDUALS OF DIFFERENT AGES

AGE	% SHOWING PERIKYMATA				% SHOWING ROD ENDS				% SHOWING CRACKS				% SHOWING STRATIFICATION			
	ALL SURFACES	BUCCAL LABIAL	LINGUAL	PROXIMAL	ALL SURFACES	BUCCAL LABIAL	LINGUAL	PROXIMAL	ALL SURFACES	BUCCAL LABIAL	LINGUAL	PROXIMAL	ALL SURFACES	BUCCAL LABIAL	LINGUAL	PROXIMAL
Unerupted*	100	100	100	100	100	100	100	100	93	96	89	93	53	48	33	64
10 to 14	100	100	100	100	99	95	100	100	93	89	95	97	57	42	60	70
15 to 19	96	93	89	100	87	80	89	93	97	93	96	100	35	18	36	50
20 to 29	96	90	98	100	87	79	87	94	95	87	98	100	18	11	24	19
30 to 39	81	75	71	96	73	59	68	95	89	84	88	96	7	4	5	13
40 to 49	62	47	47	88	53	37	42	78	85	81	89	87	4	4	6	4
Over 50	53	36	45	81	40	21	35	73	95	92	95	98	0	0	0	0

*Refers to unerupted molars from individuals of various ages; see Table I.

Two structures in addition to perikymata, rod ends, and cracks are visible on the surfaces of unerupted or recently erupted teeth. In the cervical region of about half of the surfaces, patches of material can be seen superimposed upon all other detail. These patches appear to be either the remains of an additional layer which once has covered the surface, or some stratification of the surface itself. This stratified appearance has also been noted by Wolf.[5] In general the elevated patches, which appear as depressions in the replicas, are devoid of structure, but occasionally they show faint rod end patterns. A typical region of such areas is shown in Fig. 9. While little is yet known about this stratification, it conceivably could represent remnants of the tissue described by Gottlieb as secondary cuticle.[10] Against this interpretation, however, is the fact that these areas of stratification are about as numerous on unerupted as on recently erupted teeth (Table III).

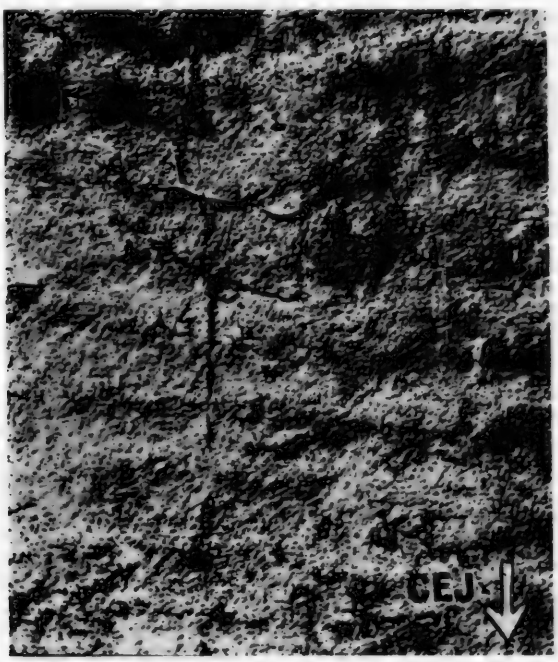


Fig. 8.—Shadowed enamel cuticle; removed from an unerupted third molar (age 25). (130×)

The cervical regions of most surfaces are also characterized by a band, generally limited to the first millimeter, in which large numbers of irregularly shaped depressions of various sizes can be seen (Fig. 7). These pits are several times larger than the enamel rod ends and are seen on both unerupted and erupted teeth. Their significance is not yet known.

DIFFERENCES IN SURFACE STRUCTURE WITH ADVANCING AGE

With the exception of carious lesions and evidence of abrasion, both general and at contact points, the most striking variations with age in the character of tooth surfaces were found in the perikymata and rod end patterns. In order to study quantitatively the differences in these structures, record was made of the presence or absence of perikymata and rod ends, of their relative prominence, and of the approximate amount of surface area showing these details. Note was also made of cracks, stratification, and any other details visible. After all the data had been recorded on punch cards, tabulations according to age, such as those of Tables III, IV, and V were made.

Fig. 9.—Surface showing stratified appearance (first premolar, mesial surface; age 18). (130×)

An impression of the differences between surfaces in individuals of different ages can be gained from consideration of the presence or absence of four structures visible on unerupted or recently erupted teeth, without reference to specific types of teeth or amounts present. Table III shows that the per cents of surfaces showing perikymata, enamel rod ends, and stratification decrease with advancing age groups, while the per cents of surfaces showing cracks remain constant. It is also to be noted that with increasing age, the per cents of proximal surfaces showing structure remain higher than those of the buccal, labial, and lingual surfaces. This would be expected since loss of structural detail apparently results from wear and those surfaces that are most protected would be expected to be at least altered.

The number, distribution, and character of the cracklike structures do not appear to vary in respect to age. This is supported by the data of Table III and by counts made of the cracks on 662 surfaces. However, the usual wide range in number on individual surfaces was noted. Cracks on old surfaces show little difference in either number or appearance (Fig. 10) from those on young surfaces (Fig. 7). This is further evidence that they may originate when a tooth is being formed.

The data of Table III show that there is a very rapid decrease with age in the per cent of surfaces on which stratified regions can be found.

Structures on surfaces of teeth from individuals of different ages are compared with those seen on surfaces of unerupted or recently erupted teeth in Table IV. To do this four arbitrary categories were established: *pronounced, medium, faint,* and *absent.*

Surfaces showing *pronounced* structure resembled the unerupted or recently erupted surfaces already described (Figs. 1, 2, 6, and 7). The persistence of marked structural detail, especially on proximal surfaces, is noteworthy.

Surfaces showing *medium* amounts of structural detail demonstrated less pronounced patterns of rod ends or perikymata and had occasional areas where no detail was to be seen (Figs. 11, 12, 13). Most structureless areas were near the occlusal or incisal edges and at the heights of contour. Perikymata on surfaces of this type usually had pronounced structure in the depths of their furrows, while the areas between were relatively smooth (Fig. 13).

Surfaces classified as showing *faint* structure had rod ends and perikymata scattered over very small areas. Their perikymata were discontinuous, and often short in length (Figs. 14, 15). Enamel rod ends were either absent, or present in small patches (Fig. 16). Most of these faint structures were seen on the cervical portions of surfaces. Figs. 3 and 4 of a preceding publication also demonstrate this type of structure.[2]

Surfaces without definite perikymata or rod ends were classified as *absent,* and showed only cracks and various defects undoubtedly due to wear. Examples of these surfaces have been shown in previous papers (Ref. 1, Figs. 1, 2, and Ref. 2, Figs. 8, 9).

It can be concluded from Tables III and IV that there is a progressive decrease in visible structure on the surfaces of teeth from individuals of increasing age, and that this decrease occurs sooner, and to a greater extent, on labial, buccal, and lingual surfaces than on proximal surfaces.

To determine how this loss of structure is related to morphological type of tooth and location in the mouth, further tabulations were made in which the teeth were grouped according to age into four morphological types: *incisors, canines, premolars, and molars.* In this case perikymata and rod ends were considered separately and were classified as pronounced and completely covering the surface (+), scattered and more faint (±)* and absent (–). Table V

*The ± group was further broken down in the original data and tabulations, but for the sake of brevity in publication, all intermediate stages of structural loss have been grouped into this single category.

TABLE IV

AMOUNT OF STRUCTURE ON TEETH FROM INDIVIDUALS OF DIFFERENT AGES

AGE	ALL SURFACES				LABIAL AND BUCCAL				LINGUAL				PROXIMAL			
	PRONOUNCED	MEDIUM	FAINT	ABSENT	PRONOUNCED	MEDIUM	FAINT	ABSENT	PRONOUNCED	MEDIUM	FAINT	ABSENT	PRONOUNCED	MEDIUM	FAINT	ABSENT
Unerupted*	100	--	--	--	100	--	--	--	100	--	--	--	100	--	--	--
10 to 14	74	24	2	0	70	23	7	0	68	32	0	0	84	16	0	0
15 to 19	47	29	22	2	16	38	43	3	46	29	18	7	76	20	4	0
20 to 29	41	23	33	3	16	28	49	7	30	21	47	2	72	21	7	0
30 to 39	24	19	42	15	6	16	60	18	22	15	40	23	48	29	20	3
40 to 49	8	12	48	32	1	6	48	45	0	2	51	47	20	27	45	8
Over 50	4	11	45	40	1	6	34	59	3	7	45	45	9	23	60	8

*Refers to unerupted molars from individuals of various ages: see Table I.

Fig. 10.

Fig. 11.

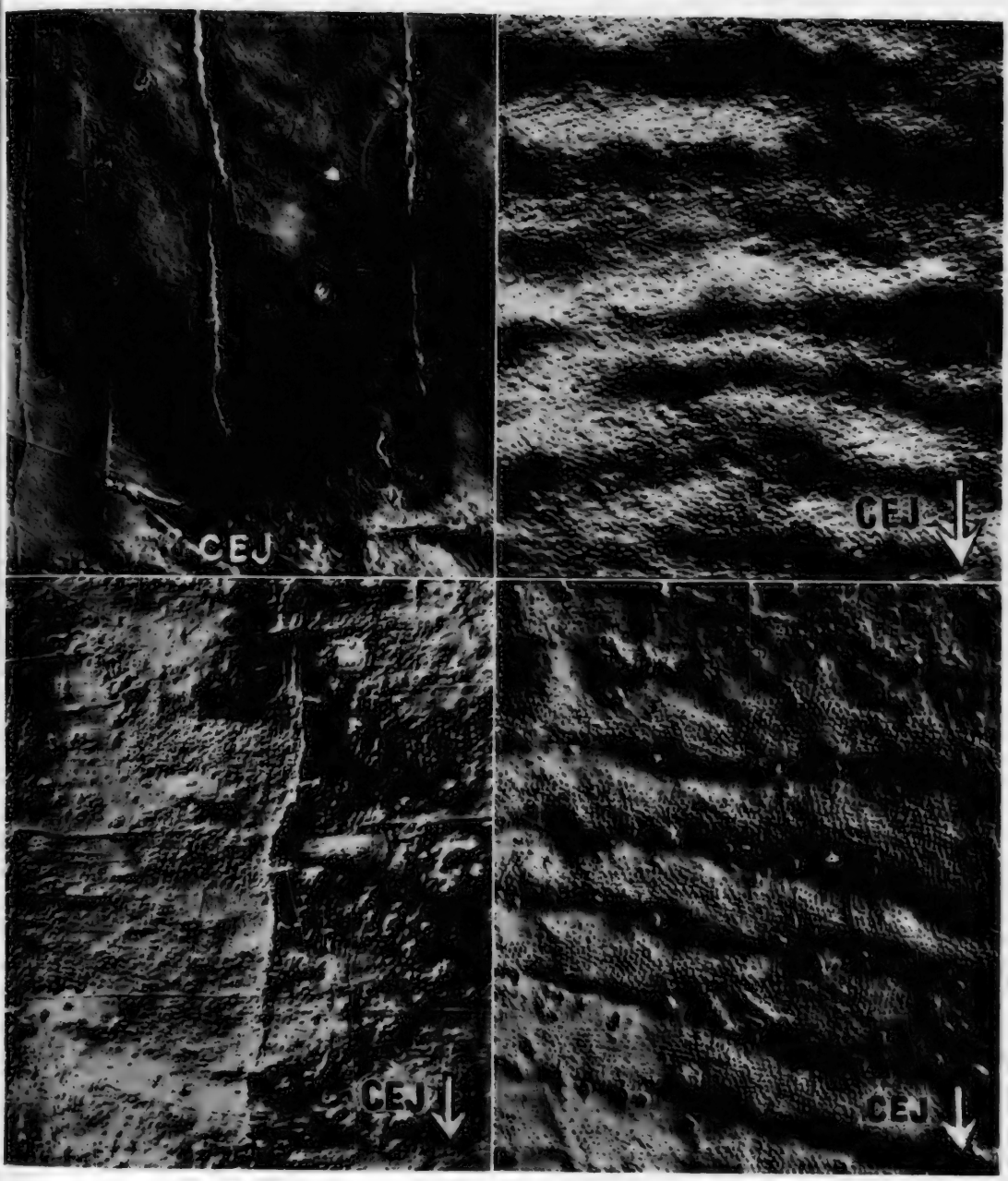


Fig. 12.

Fig. 13.

Fig. 10.—Cracks on the surface of an old tooth (first molar, buccal surface; age 50). (130×)

Fig. 11.—Surface showing *medium* amount of structural detail (second premolar, buccal surface; age 25). (130×)

Fig. 12.—Surface showing *medium* amount of structural detail (second molar, lingual surface; age 25). (130×)

Fig. 13.—Surface showing *medium* amount and structural detail (first molar, mesial surface; age 52.) (130×)

shows that the buccal and lingual surfaces of molars and premolars retain more structure with advancing age than do similar surfaces on canines and incisors. This is also the case with the proximal surfaces, but, in all types of teeth, structure on these surfaces persists much longer than on labial, buccal, and lingual surfaces.

From the results of these investigations it is possible to deduce the probable sequence of loss of detail on tooth surfaces. The first evidence of loss of structural detail occurs on the buccal, labial, and lingual surfaces as a weakening of the enamel rod-end pattern. Next, individual rod ends and patches of rod ends begin to disappear, and more slowly the perikymata on the occlusal or incisal portions of surfaces become less pronounced. The last regions to lose their structural detail are the cervical thirds of surfaces. The portions of some proximal surfaces occlusal to contact points show similar loss of detail. Otherwise, proximal surfaces retain pronounced patterns of perikymata and rod ends until late in tooth life (Table V). Contact points are the only areas on proximal surfaces showing visible wear at early ages. Signs of initial wear at these points were observed on 55 per cent of the replicas from premolars in the 10-to-14-year age group, and on 70 per cent in the 15-to-19-year age group.

It appears that cuticle is present on those surfaces of teeth from older age groups which show pronounced patterns of perikymata and rod ends. Twenty-five such surfaces were chosen, and typical membranes were floated from each. This suggests that the presence of pronounced patterns of perikymata and rod ends on older teeth resembling those seen on unerupted or recently erupted teeth is indicative of the continued presence of enamel cuticle.

SURFACES ON CARIOUS TEETH

As stated earlier in this paper, no differences have yet been seen between the surfaces of noncarious teeth, noncarious surfaces of carious teeth, and the intact portions of carious surfaces. A number of the replicas taken from carious surfaces showed small, untreated lesions. Some of these were discrete, others were surrounded by areas of decalcification. In all cases the areas of intact tooth surface immediately adjacent to the affected regions appeared like normal noncarious surfaces in that particular age group.

Carious lesions occurring on the surfaces of teeth within a few years after eruption often border areas which, showing pronounced patterns of perikymata and rod ends, presumably are covered by cuticle (Fig. 17). This gives rise to questions as to whether or not cuticle actually serves a protective function, as postulated by some,[10, 11] and whether or not there are natural or acquired defects in cuticle which predispose these areas to caries. These questions cannot be answered without a thorough study of the earliest manifestations of caries.

DISCUSSION

Optical studies have been undertaken as preliminary to the electron microscopic investigation of tooth surface structures. The results of this study emphasize the dangers in interpreting electron micrographs of randomly selected tooth surfaces before an adequate preliminary survey has been made at lower magnifi-

TABLE V

STRUCTURE OF TEETH OF DIFFERENT MORPHOLOGICAL TYPES

AGE	LABIAL AND BUCCAL						LINGUAL						PROXIMAL					
	PERIKYMATA			ROD ENDS			PERIKYMATA			ROD ENDS			PERIKYMATA			ROD ENDS		
	%+	%±	%-	%+	%±	%-	%+	%±	%-	%+	%±	%-	%+	%±	%-	%+	%±	%-
Incisors																		
Unerupted*																		
10 to 14																		
15 to 19	50	50	0	0	33	67												
20 to 29	25	69	6	0	62	38	0	100	0	0	67	33	73	27	0	13	73	14
30 to 39	8	50	42	0	25	75	0	29	71	0	29	71	43	43	14	0	71	29
40 to 49	3	31	66	0	33	67	0	22	78	0	22	78	17	50	33	4	50	46
Over 50	2	21	77	0	11	89	0	13	87	0	25	75	18	50	32	0	68	32
Canines																		
Unerupted*																		
10 to 14																		
15 to 19																		
20 to 29	33	67	0	0	100	0							100	0	0	0	67	33
30 to 39	33	50	17	0	51	33							33	67	0	0	100	0
40 to 49	10	37	53	0	37	63	0	50	50	0	50	50	31	63	6	0	79	21
Over 50	4	17	79	0	8	92	0	17	83	0	27	73	42	50	8	0	58	42
Premolars																		
Unerupted*																		
10 to 14	96	4	0	52	46	2	97	3	0	36	64	0	100	0	0	84	16	0
15 to 19	52	48	0	0	91	9	62	38	0	0	100	0	86	14	0	57	39	4
20 to 29	41	41	18	0	73	27	24	70	6	0	76	24	100	0	0	33	67	0
30 to 39	23	44	33	0	56	44	20	53	27	0	53	47	77	23	0	8	84	8
40 to 49	0	36	64	0	9	91	0	60	40	0	30	70	75	25	0	25	75	0
Over 50	0	53	47	0	21	79	8	54	38	0	15	85	58	33	9	8	75	17
Molars																		
Unerupted*	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0	93	7	0
10 to 14	75	25	0	13	87	0	100	0	0	21	79	0	89	11	0	67	33	0
15 to 19	57	29	14	29	57	14	88	6	6	63	37	0	100	0	0	81	19	0
20 to 29	64	32	4	14	82	4	70	30	0	43	57	0	89	11	0	31	67	2
30 to 39	41	44	15	9	66	25	47	34	19	22	64	19	82	15	3	48	46	6
40 to 49	18	64	18	0	59	41	0	67	33	0	60	40	52	48	0	19	71	10
Over 50	47	32	21	0	68	32	10	65	25	0	65	35	63	32	5	5	79	16

*Refers to unerupted molars from individuals of various ages; see Table I.

Fig. 14.

Fig. 15.

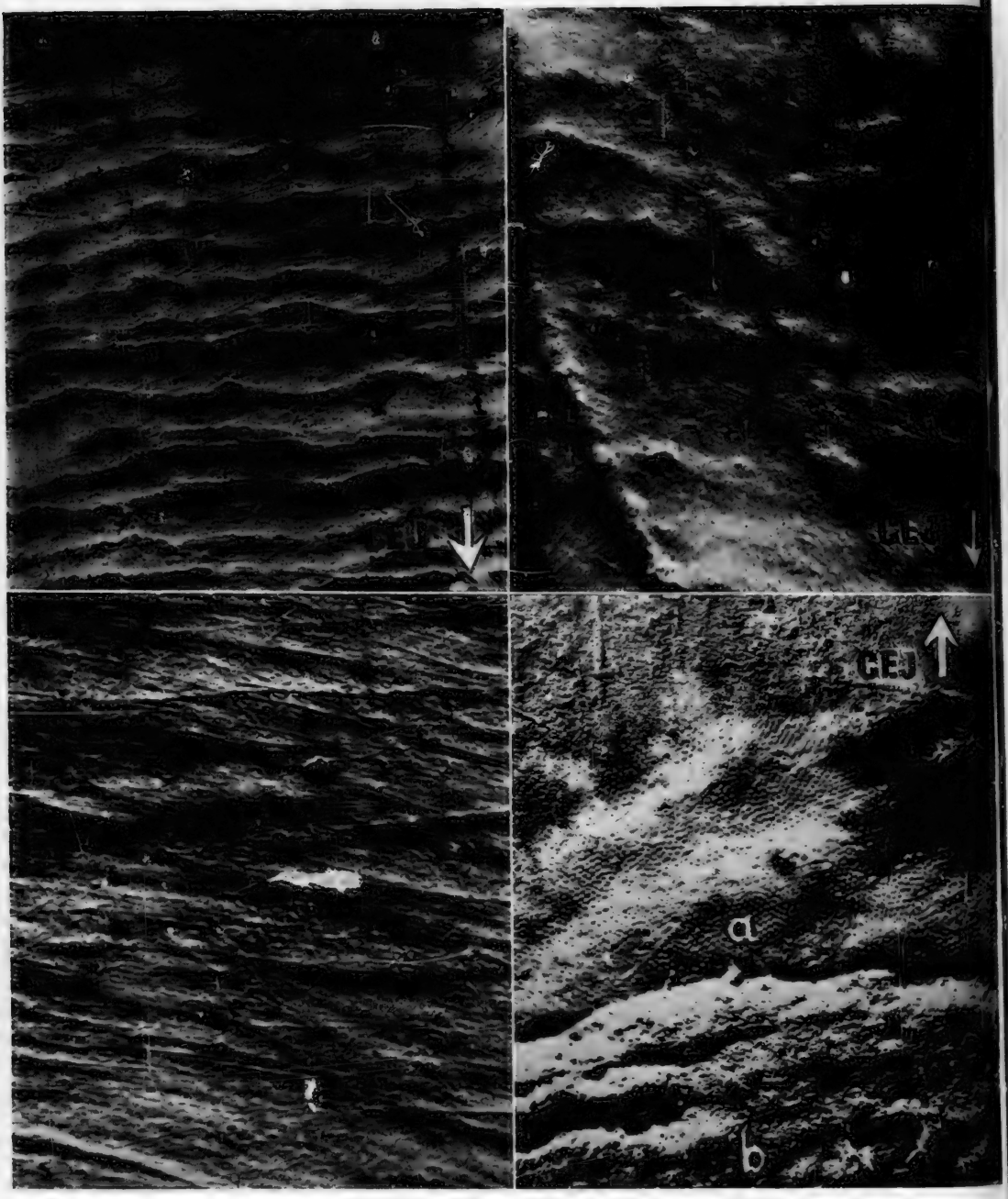


Fig. 16.

Fig. 17.

Fig. 14.—Surface showing *faint* structural detail (first premolar, distal surface; age 63). (130×)

Fig. 15.—Surface showing *faint* structural detail (canine, labial surface; age 63). (130×)

Fig. 16.—Surface showing *faint* structural detail (second molar, buccal surface; age 60). (250×)

Fig. 17.—Surface *a* immediately adjacent to carious lesion *b* (first molar, distal surface; age 24). (130×)

cations. It now appears necessary that the age, morphological type of tooth, and position of surface on the tooth be known. Furthermore, there is so much detail on surfaces that optical microscopy is required to give proper identification to the structures seen in small areas at high magnification with the electron microscope.

The fact that recently erupted teeth are completely covered with cuticle and the persistence of this cuticle over large areas of the tooth surfaces of older individuals raise doubt regarding the extent to which the properties of tooth surfaces can be considered analogous to those of internal enamel. It is hazardous to assume, for example, that the action of chemical agents upon tooth surfaces is the same as on powdered enamel or ground sections. Further studies should be made of the properties of cuticle-covered surfaces and their reactions and permeability to chemical agents. This is especially needed for an understanding of the action of those agents for the control of caries which are being applied at ages when cuticle is present.

SUMMARY

A description is given of structural details on the surfaces of unerupted and recently erupted teeth as visualized on metal-shadowed collodion replicas. The changes in these details that occur with advancing age are demonstrated through tabular and descriptive data.

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THE EFFECT OF HYPOPHYSECTOMY UPON THE PERMANENT DENTITION OF RHESUS MONKEYS

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THE purpose of this study was to observe the effect of hypophysectomy on the rate of growth and the extent of calcification of dentin in young (about 2 years old) Rhesus monkeys.

Schour[1] and Schour and Hoffman[2, 3] published the results of their experiments on these and other animals, establishing the normal rate of dentin apposition at about 4 μ per day, and the calcification rhythm at about 16 μ per day. This latter estimation was based upon the measurement of the width of pairs of light and dark incremental layers. Their technic consisted of injections of alizarin red S (Coleman and Bell) at varying intervals of time, a technic first modified for tooth study by Gottlieb[4] and later used extensively by Cameron.[5] This dye was taken up by the blood stream and deposited in the newly formed dentin. On sectioning the teeth, the alizarin dye was seen as red lines at varying of distances in the dentin, one red line for each injection. By measuring the distance between the red lines and dividing by the number of days which elapsed between injections, the daily rate of dentin apposition was determined. They established, as well as the daily rate of dentin apposition, the fact that there is a gradient or pattern in the rate of growth in different parts of a tooth, and the calcification rhythm remains at about 16 μ per day regardless of the rate of dentin apposition.

METHODS

For the purpose of establishing a control for our operated series, Schour's and Schour and Hoffman's studies were repeated. While only one control animal is reported here, several others were used for this purpose.

Six animals comprise the group reported here. In all six, attempts at hypophysectomy were made. The operation was successful in five. In one, the sphenoid could not be reached because the odontoid process was excessively protuberant. The latter animal served as an operated control. Of the five successfully operated animals, the removal of the hypophysis was complete in two and incomplete in three, as established by serial sections post mortem. Details are to be noted in Table I.

The operations were carried out under Nembutal anesthesia by the parapharyngeal approach after the technic devised by Phillip E. Smith.[6] The operations were performed by Drs. Dale Tyndal and E. F. Scowen, who generously

Received for publication August 17, 1948.

*Deceased.

TABLE I
HYPOPHYSECTOMY OF FEMALE MONKEYS

MONKEY NUMBER	FIRST WEIGHT PRE-EXPERIMENTAL (GRAMS)	LAST WEIGHT BEFORE ANIMAL SACRIFICED (GM.)	EXPERIMENTAL PROCEDURE		LENGTH OF EXPERIMENT IN DAYS	DAYS HYPOPHYSECTOMIZED	DAILY APPOSITION OF DENTIN IN MICRA OCCLUSAL	ESTIMATION OF RETARDATION OF DENTIN APPOSITION AFTER OPERATION (%)
			OPERATION	TOTAL OF DAILY INJECTIONS				
487 ♀	2450	1700	Hypophysectomy complete	Eschatin 537 dog units total Gonadin 5675 Rat units	40	32	1.5	63
488 ♀	2350	1910	Hypophysectomy complete	Eschatin 1100 dog units Squibb Ant. Pit. Growth Hormone 3585 units	86	80	1.8	54
492 ♀	2000	1800	Hypophysectomy attempted. Unsuccessful	None	147	—	4.1	—
500 ♀	2550	1960	Hypophysectomy. Incomplete	Eschatin 1725 dog units total	74	69	2.6	36
501 ♀	2350	2300	Hypophysectomy. Incomplete	Eschatin 2450 dog units total	112	98	2.5	39
501-A ♀	2300	2320	Hypophysectomy. Incomplete	Eschatin 2950 dog units	236	171	1.8	54

contributed the heads of the monkeys for this study. The animals were given intraperitoneal injections of 2 per cent alizarin red S (Coleman and Bell) at intervals before and after hypophysectomy.

Certain hormones were also injected subcutaneously, daily. A hormone extracted from the adrenal cortex (Eschatin: Parke-Davis) was used for the purpose of maintaining the animals in better condition than would otherwise be the case during experimental conditions. Another hormone, gonadotropic in nature, extracted from mare serum (Gonadin: Cutter) was given to one animal to observe the effect of the estrogenic hormone on the growth of dentin. Another animal was given a preparation made from the pituitary gland containing the growth factor (Growth Hormone: Squibb) to see if growth of dentin could be stimulated in this way.

Particular attention was given to the diets of the animals (Purina Dog Chow) to rule out known factors which might detract from the validity of the results. The animals were weighed often and care was exercised to see that the food given was consumed.

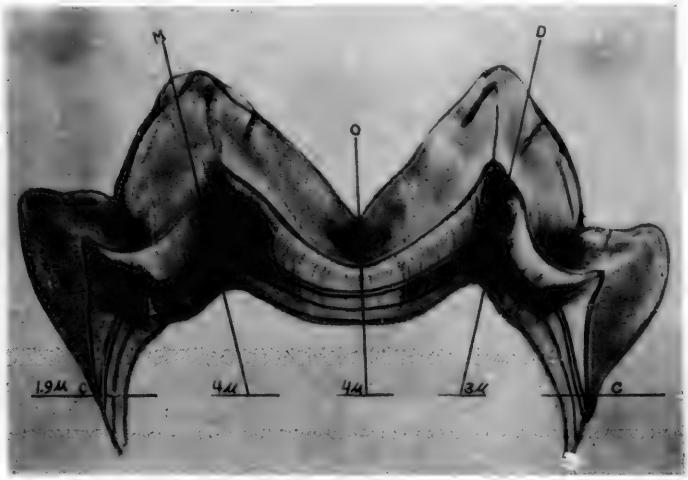

Fig. 1.—Monkey No. 492. Normal control, mesiodistal ground section. Mag. 20×. This photomicrograph shows the rate of dentin apposition by four alizarin lines. Ten days has elapsed between lines number one and two, 67 days between lines number two and number three, and 70 days between lines number three and number four. All measurements are taken along the direction of the dentinal tubules. *O*-occlusal, *M*-mesial, *D*-distal, and *C*-cervical. The figures show the average daily rate of dentin apposition.

After the animals were killed, ground sections of some of the teeth were made. Measurements were made with an eyepiece micrometer after the manner already described.[3] While for the sake of easy comparison only one standard measurement on the lower second molar, unerupted, is reported here, many other measurements were made, both in other areas of this tooth and in other teeth. They are not reported because the results are all of the same order. All measurements were made along the dentinal tubules.

FINDINGS

The average daily rate of dentin apposition in the controls was about 4 μ per day. The calcification rhythm was about 16 μ per day, and the gradients already mentioned were also observed. Thus along the line *O* in Fig. 1, the rate of growth was 4.1 μ per day; at the mesial line (*M*) it was 4.3 μ per day; and at the cervical line (*C*) it was 2 μ per day. The rate of growth is fastest at the dentino-enamel junction, slowing up somewhat until it reaches what is finally the pulp chamber in the completed tooth. The rate is not the same in all teeth of the same animal, nor in different animals, but the figure of 4 μ per day is fairly representative of the controls. (See Table II.)

TABLE II

EFFECT ON DENTIN APPOSITION IN DEVELOPING UNERUPTED TEETH OF YOUNG RHESUS MONKEYS AFTER HYPOPHYSECTOMY

MONKEY	DAYS HYPOPHY-SEC-TOMIZED	DAYS EXPERI-MENT	TREATMENT	DAILY APPOSITION OF DENTIN IN MICRA		RETARDATION OF DENTIN APPOSITION AFTER HYPOPHYSECTOMY	
				CERVICAL	OCCLUSAL	CERVICAL (%)	OCCLUSAL (%)
492		147	None	2.0	4.1		
487	32	40	Hypophysectomy Eschatin Gonadin	1.3	1.5	35	63
488	80	86	Hypophysectomy Eschatin Phyone	1.6	1.8	20	54
500	69	74	Hypophysectomy Eschatin		2.6		36
501	106	112	Hypophysectomy Eschatin	1.3	2.5	35	39
501-A	171	236	Hypophysectomy Eschatin	2.0 P.E.	4.0 P.E.		
				1.3	1.8	35	54
Average retardation of dentin apposition in experimental group						31	49

In our hypophysectomized group marked retardation in the daily rate of dentin apposition was found resulting from this operation. The calcification rhythm and the pattern of growth were also affected. Thus, while the retardation at the occlusal line (*O*) was 63 per cent in Monkey #487, at the cervical line (*C*) it was only 35 per cent (Fig. 2 and Table II).

Schour and Hoffman reported that hypophysectomy does not interfere with the 16 μ rhythm in rat incisors. In photomicrograph of Monkey #487 (Fig. 3.), the contour lines of Owen constituting the calcification rhythm may be seen. It appears on comparison with the stage micrometer photographed at the same magnification that the width of pairs of these bands is not uniform, and therefore we suggest that the observation of Schour on the uniformity of calcification rhythm needs further clarification.

In this study the amount of retardation was greatest in the animals in which the hypophysectomy was complete. Thus in Monkey #487 (Fig. 2), retardation of about two-thirds is observed, that is, the dentin grew at about one

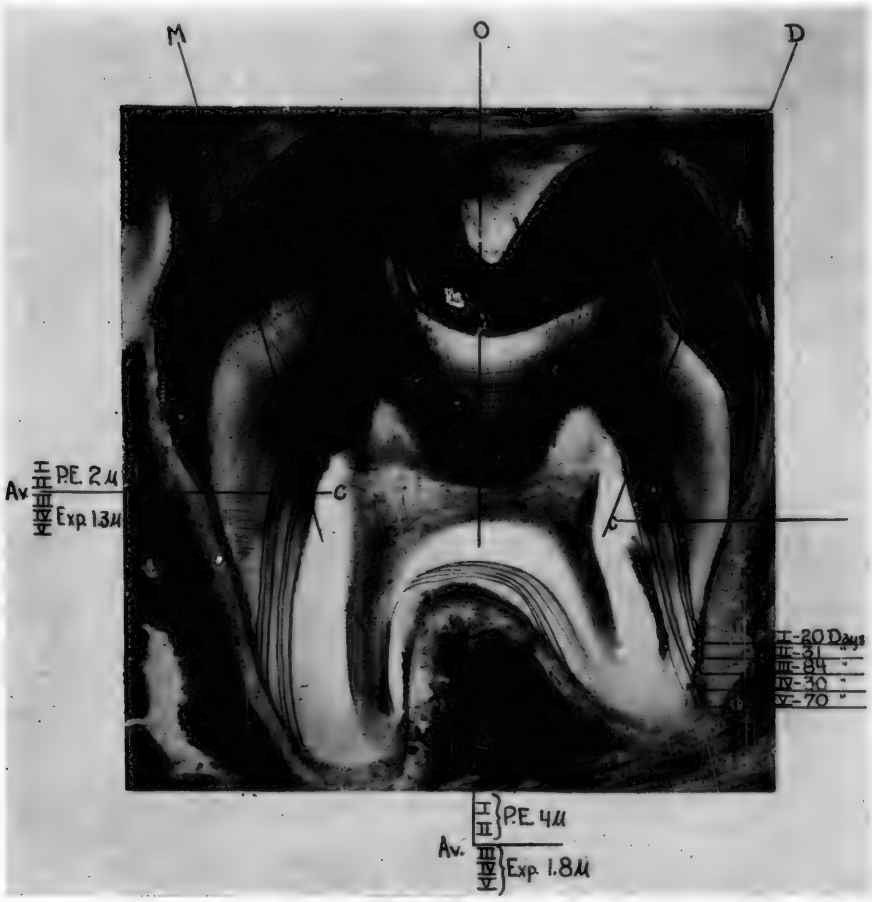

Fig. 2.—Monkey No. 487. Mesiodistal ground section. (Mag. 20×.) This shows the rate of dentin apposition by six alizarin lines. Twenty days have elapsed between lines *I* and *II*; 31 days between *II* and *III*; 84 days between *III* and *IV*; 30 days between *IV* and *V*; and 70 days between *V* and *VI*. All measurements are taken along the direction of the dentinal tubules. *M*-mesial, *D*-distal, *O*-occlusal, *C*-cervical, *P.E.*-pre-experimental, and *Exp.*-post-experimental. The figures show the average rate of dentin apposition measured at the occlusal and cervical areas.

third its normal rate. In Monkey #488, the retardation of dentin growth was about one-half; in Monkey #500 and Monkey #501, the retardation was about one-third. Both of these animals had incomplete removal of the hypophysis.

In Monkey #501-A, the amount of retardation was about one-half. This was established by comparing pre-experimental with postoperative measurements. The removal of the hypophysis in this animal was also incomplete. How-

ever, more of the gland was removed from this animal than from the other two animals in which the hypophysis removal had been incomplete. From these three incomplete removals, it might appear that the amount of retardation is somewhat proportional to the amount of gland removed. However, considering the limited number of observations, this must be advanced as an approximation.

Fig. 3.—In the higher magnification of an incisor tooth of Monkey No. 487, the contour lines of Owen, constituting the calcification rhythm, can be observed. Upon comparison with the stage micrometer photographed at the same magnification, it may be noted that the width of pairs of these bands is not uniform throughout their length.

In Monkey #501-A, the tooth is in an advanced state of development over the corresponding teeth of the other two animals, where normally there would be some retarded growth. However, in comparing the actual rate of development with control teeth in more comparable situations, it is observed that retardation did occur.

Soft X-ray (Grenz ray) study of these teeth (Fig. 4) showed that they were of normal calcification density, quite unlike the results obtained by thyroidectomy in monkeys (Ziskin and Applebaum).[7]

Whether the estrogenic hormone plays a role in the rate of growth of dentin could not be determined in this study.

CONCLUSIONS

From the experimental study cited here, the following conclusions may be drawn: (1) The normal rate of dentin apposition in the Rhesus monkey is about 4 μ per day. (2) The rate of growth differs in the various parts of a tooth. (3) The calcification rhythm is about 16 μ per day. (4) Hypophysectomy causes (a) severe retardation in the rate of dentin apposition, (b) the gradient to be affected, and (c) the calcification rhythm to be affected. (5) The calcification of the enamel and dentin are of normal density, unlike that produced by thyroidectomy.

A. *B.*

Fig. 4.—*A*, This is a photomicrograph of a labiolingual ground section of a developing incisor crown from Monkey No. 487. *B*, Grenz ray negative of the same ground section showing normal calcification density gradient of enamel and dentin. This picture is quite unlike that observed in teeth of thyroidectomized monkeys.

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ALKALINE PHOSPHATASE IN PULPAL TISSUE OF HUMAN TEETH

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INTRODUCTION

THE enzyme, or enzyme entities, known as alkaline phosphatase has been the subject of considerable investigation because of its relation to carbohydrate metabolism and to the process of calcification. The Gomori[1, 2, 3] and Takamatsu[4] technics, by which the presence of this enzyme may be visualized in tissue sections, have given added impetus to studies in this field, and numerous tissues, both normal and pathological, have been investigated. Developing teeth have received their share of attention in regard to alkaline phosphatase, but nearly all of the dental material studied has been derived from mammalian sources other than human. The most stringent factor limiting the investigators' choice of material is the fact that routine methods of decalcification adversely affect alkaline phosphatase and prevent its demonstration by means of the Gomori method.

The objectives of the present investigation are: (1) to confirm the presence and localization of alkaline phosphatase in human pulpal tissue during dentinogenesis, and (2) to ascertain the success with which the technic of demonstrating alkaline phosphatase in decalcified tissues, as described by Lorch in her earlier work,[5] could be applied to dental tissues. Recently Greep, Fischer, and Morse[7] have presented a detailed report of their investigations in which they have demonstrated the enzyme in decalcified rodent material, using a method somewhat different from that of Lorch.[5, 6]

MATERIAL AND METHODS

The material for this study was obtained from a human term fetus. Segments of the maxilla were fixed in 95 per cent alcohol and the teeth dissected from their crypts.

For the purpose of demonstrating alkaline phosphatase in undecalcified tissues, a deciduous canine, with crown about one-quarter completed, was fixed, dehydrated, and embedded in paraffin. The sections suffered some distortion but certain observations could be made.

To demonstrate alkaline phosphatase in tissue decalcified for varying periods of time, a deciduous second incisor was decalcified for 9 days, two deciduous first incisors for 12 and 16 days, respectively, and a deciduous molar for 23 days. The method of decalcification is based on one presented by Lorch[5] and was carried out as follows:

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After fixation the teeth were carried through descending grades of alcohol to water and placed in the following decalcifying solution for the desired length of time:

0.2 N acetic acid 0.2 N sodium acetate	adjusted to pH 4.5
Zinc sulfate	0.004 Gm./500 c.c. of solution

The entire procedure from fixation to the completion of decalcification was carried out at a temperature of about 5 degrees Centigrade. The decalcifying solution was changed daily.

When decalcification was halted, the tissues were washed well. Reactivation of the enzyme was carried out by incubating the teeth at 37° C. for from 2 to 4 hours in the following solution:

1.0 per cent sodium barbital	1 volume
0.075 per cent glycine	2 volumes

Subsequent to incubation the tissues were washed thoroughly, dehydrated, and embedded in paraffin.

Except for one specimen, each tooth was cut at right angles to its future long axis, starting at the cervical region of the developing crown and continuing incisally as far as possible. The sections were cut at 7 micra, attached to slides, and treated by a modified Gomori technic for the demonstration of alkaline phosphatase. The incubating medium specified by Gomori[3] is as follows:

2 per cent sodium glycerophosphate	12.5 c.c.
2 per cent sodium barbital	12.5 c.c.
2 per cent calcium chloride	2.5 c.c.
2 per cent magnesium sulfate	1.0 c.c.
distilled water	25.0 c.c.
chloroform	a few drops

Control slides were treated at all stages of the various procedures in the same manner as test slides except that the substrate, sodium glycerophosphate, was omitted from the above mixture. The most satisfactory incubation period was from 3 to 5 hours at a temperature of 37 degrees Centigrade. The slides were then rinsed briefly and placed in 2 per cent cobalt acetate for 15 to 30 minutes. Next, after thorough rinsing, the slides were placed in a dilute aqueous solution of ammonium sulfide, and a black or brown cobalt sulfide precipitate formed wherever phosphates of calcium were present.

The slides were then washed, dehydrated, cleared in xylol, and mounted. Counterstains may be used if desired but none were employed in the sections under discussion.

FINDINGS

1. Undecalcified canine (Figs. 1, 2, and 3):

A. Control sections: The calcified dentin appears black whereas the predentin, odontoblasts, pulpal cells, and intercellular substance are free of a precipitate and are clear.

B. Test sections: Again the dentinal matrix appears black or gray. The predentin is free of precipitate except along the pulpal margin. A heavy precipitate indicative of the presence of alkaline phosphatase is present in the odontoblasts and their processes. The subodontoblastic area is strongly phos-

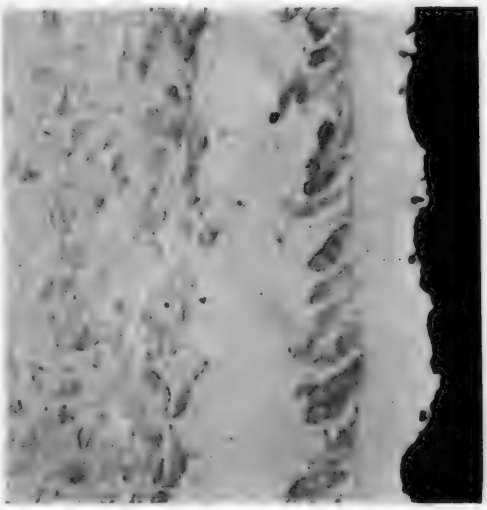

Fig. 1.—Control section of undecalcified canine. Dentin black. Predentin and odontoblasts free of precipitate. Pulpal tissue, separated from former structures, also is free of a precipitate. ×575 Orig. Mag.

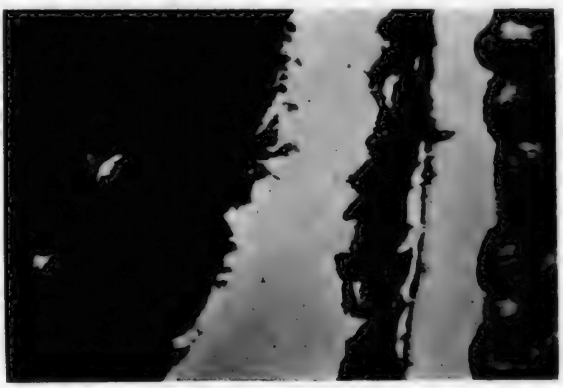

Fig. 2.—Test section of undecalcified canine. Dentin black. The predentin is clear except along pulpal margin. Note intense precipitate in odontoblasts and pulpal tissue indicative of phosphatase activity. Several odontoblastic processes are visible. The pulpal tissue is separated from the odontoblasts, predentin, and dentin as in Fig. 1. ×575 Orig. Mag.

phatase positive. The enzyme is also found in and about the walls of blood vessels throughout the pulp. The pulpal cells, other than the odontoblasts, react with various degrees of intensity. The enzyme appears to be distributed throughout the intercellular substance.

2. Decalcified first and second incisors (Figs. 4 and 5):

A. Decalcified for 9 days: The dentinal matrix appears pale gray, and the walls of the dentinal tubules are emphasized. The predentin is clear although a precipitate may be found for a short distance in some of the tubules. The odontoblasts are phosphatase positive and the pulpal cells present an intense reaction. The intercellular reaction is diminished and the walls of blood vessels are negative, or nearly so, for phosphatase. A reaction for the enzyme may be found in the lumen of smaller vessels.

B. Decalcified for 12 and 16 days: The findings are essentially the same as in the specimen decalcified for 9 days except that the reaction is less intense. The lumina of vessels are free of phosphatase.

C. Control sections: All cell types and the predentin are free of a precipitate. The dentin appears as described in paragraph 2A.

3. Decalcified deciduous molar:

A. Decalcified for 23 days: Little enzyme activity remains. Only a few cells in the odontoblastic region respond.

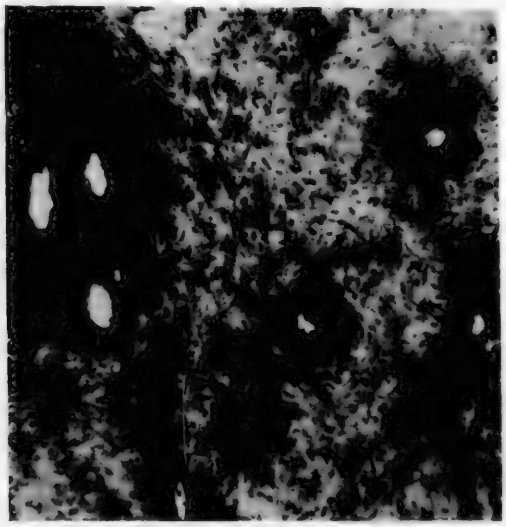

Fig. 3.—Test section of undecalcified canine. Note phosphatase activity about the vessels of the pulp, and the varying intensity of cellular reaction. ×250 Orig. Mag.

DISCUSSION AND CONCLUSIONS

Investigations by Morse and Greep,[7, 8] Horowitz,[9] and Bevelander and Johnson,[10, 11] have shown that alkaline phosphatase is abundant in pulpal tissue of teeth in which dentinogenesis is imminent or in progress. Areas of especial abundance were shown to be in the odontoblastic and subodontoblastic regions as well as in the odontoblasts themselves. Although Gomori,[3] and Engel and Furuta[12] reported odontoblasts primarily negative in reaction for phosphatase, all found the enzyme to be present in the subodontoblastic region.

The present investigation confirms the presence of the enzyme in human pulpal tissue of teeth undergoing dentin formation and calcification, and it agrees with the findings of Greep, Fischer, and Morse, Horowitz, and Bevelander and Johnson in regard to localization.

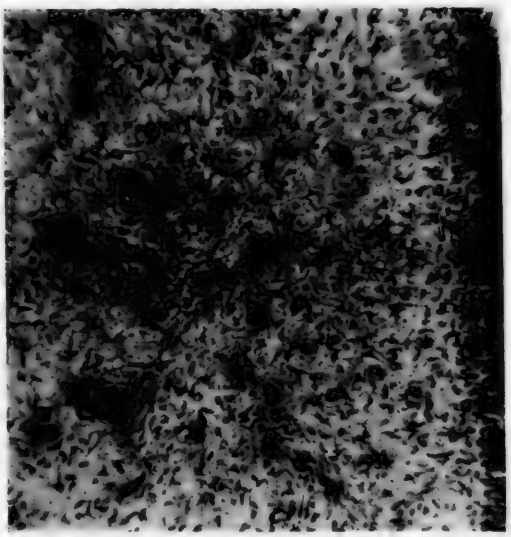

Fig. 4.—Test section of second incisor decalcified for 9 days. Odontoblasts and pulpal cells positive for phosphatase, the latter with consistent intensity. Intercellular localization of the enzyme diminished. Note precipitate in lumina of vessels. ×230 Orig. Mag.

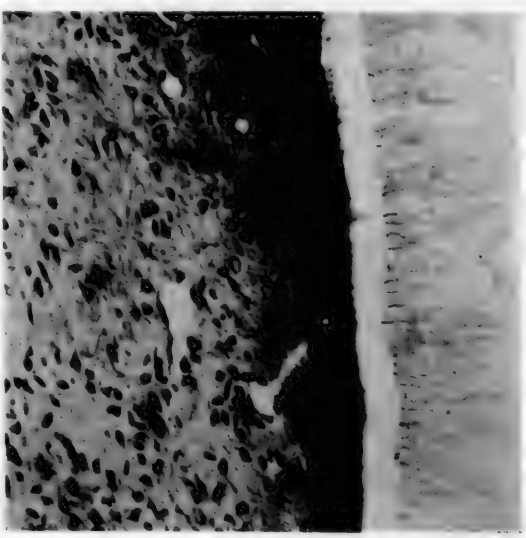

Fig. 5.—Test section of first incisor decalcified for 12 days. Dentin pale gray, tubule walls emphasized. Predentin clear except for precipitate in some tubules. Odontoblasts phosphatase positive as are other cellular elements of the pulp. Note capillaries with lumina clear, endothelial nuclei sharply revealed. ×300 Orig. Mag.

Alkaline phosphatase may be demonstrated in pulpal tissue of young teeth subjected to an acetate decalcifying solution for a period of about 16 days at pH 4.5 in the presence of zinc ions. The intensity of the reaction diminishes as decalcification is continued.

By comparing decalcified with undecalcified sections, it can be observed that in addition to a decrease in enzyme activity, the precise localization of alkaline phosphatase is altered by the decalcifying-reactivating procedure. In Fig. 3 the pronounced phosphatase activity about blood vessels is evident. The presence of many pulpal cells is revealed primarily by the precipitate formed on the nuclear membrane since the cytoplasm and, to a great extent, the karyoplasm are clear. On the other hand, a consistently strong cellular reaction is observed in the pulpal tissue of decalcified teeth while the perivascular and intercellular precipitate is diminished in these specimens. The nature and mechanism of this modified localization require clarification.

The work of Robison,[13] Fell and Robison,[14] Gomori,[3] and others, indicate that phosphatase is associated with calcification of cartilage and bone. Although Roche[15] and Nuckolls, Leicester, and Dienstein[16] have adduced theoretical mechanisms of calcification, the exact relation of phosphatase-rich pulpal cells and tissue areas to the phenomenon of dentin calcification is speculative and requires further chemical and histological investigation.

The author is indebted to Dr. Samuel W. Chase for aid and helpful criticism given during the course of this investigation.

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CALCIFICATION OF TEETH

II. Comparison of Some Properties of Human and Rat Teeth

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THE purpose of these investigations was to study and compare the composition and structure of rat and human teeth and the changes due to fluoride treatment. The correlation of the properties of human teeth to those of the upper incisors of the rat is of interest because the composition of the latter was correlated to blood and diet.[1] The influence of sodium fluoride is also of interest because of the importance attached to the fluoride ion in preventing dental caries in both man and lower animal.[2, 3]

Powder diagrams were taken before and after ignition at 900° as a further means of differentiation. Earlier studies have shown that high phosphate bones give β-$Ca_3(PO_4)_2$ patterns instead of the usual apatite patterns on ignition.[4]

Saturated sodium fluoride was used to insure maximum changes and the experiments were controlled by equilibrating with distilled water adjusted to the same pH.

EXPERIMENTAL

Rat teeth were obtained from albino rats raised in our laboratory from an original Wistar strain. The mother and young were kept on the stock diet of Bills, Honeywell, Wirick, and Nussmeier.[5] The upper incisors used were from rats sixty to seventy days of age. This age was chosen to correspond to the age of animals kept on our experimental diets in the study where a correlation had been shown between the composition of blood and diet.[1]

Human teeth were caries-free molars carefully selected from teeth extracted in the course of dental practice.

Treatment of Dentin and Enamel.—The teeth were defatted, ground to pass a sixty-mesh sieve, and the enamel and dentin then separated. A portion of the enamel and dentin was analyzed for calcium, phosphate, and carbonate. Portions of the enamel and dentin, in glass-stoppered tubes, were shaken with 3.0 ml. of a saturated sodium fluoride solution for sixteen hours, and other portions were shaken with 3.0 ml. of distilled water at the same pH (7.1) at the same time. Approximately five mg. samples of enamel and fifty mg. samples of dentin were used for analysis and for equilibration. The samples were centrifuged and the

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*The x-ray diffraction studies were taken from the thesis of Howard A. Kirshner submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry at the Polytechnic Institute of Brooklyn, June, 1948.

supernatant liquid removed by aspiration. The residues were washed once with distilled water at a pH of 7.1 and the washings discarded. The residues were then analyzed for calcium, phosphate, and carbonate.

The methods employed in separation and analysis of the enamel and dentin were those referred to and described in our previous paper.[1]

X-ray Diffractions.—Portions of all specimens were ignited at 900° for approximately two and one-half hours. Powder diffraction patterns were made on small amounts of both ignited and unignited specimens, ground to pass a 250-mesh sieve, by means of a 57.3 mm. Debye-Scherrer camera. The samples were mounted in thin-wall capillaries and nickel-filtered copper radiation was used. Exposures were of three and one-half hour duration.

Calculation of Chemical and X-ray Data.—The composition was calculated from the data in the manner described in the previous paper.[1] The *d*-spacings were calculated from the x-ray plates by the method described by Bragg.[6]

COMPOSITION OF TEETH BEFORE AND AFTER EQUILIBRATION WITH SODIUM FLUORIDE AND WATER

It is seen from Table I that most of the composition changes were in the phosphate content both for human and rat dentin and enamel. These changes were more pronounced under the influence of sodium fluoride than under water alone. Changes in calcium content are evident under the influence of water in rat dentin and human enamel, but are not evident with sodium fluoride. No significant changes in carbonate content occurred.

TABLE I

COMPOSITION OF TEETH BEFORE AND AFTER EQUILIBRATION WITH NaF AND H_2O AT pH OF 7.1
(Absolute values per samples analyzed)

	TREATMENT								
	NONE			WATER			SODIUM FLUORIDE		
	Ca mMx100	PO_4 mMx100	CO_3 mMx100	Ca mMx100	PO_4 mMx100	CO_3 mMx100	Ca mMx100	PO_4 mMx100	CO_3 mMx100
Rat									
Enamel	4.77	3.24	0.31	4.75	2.64	0.30	4.81	2.28	0.31
Dentin	33.8	28.1	2.28	30.8	22.6	2.20	34.6	21.2	2.16
Human									
Enamel	4.60	2.51	0.25	4.50	2.48	0.23	4.54	2.24	0.25
Dentin	34.0	19.7	2.79	33.8	19.4	2.73	34.0	18.1	2.76

Enamel and dentin changes cannot be fully compared since fifty mg. of dentin and only five mg. of enamel were used in the presence of the same amounts of supernatant fluid. In spite of this, one is impressed by the larger changes in the phosphate rat dentin than that of the enamel. No corresponding differences can be observed between human enamel and dentin. The explanation may be either in the different amount of active surface exposed, or that rat dentin is more readily attacked under these conditions. Greater changes were observed in the rat teeth than in the human teeth. Explanation of these changes cannot be given until these studies are carried out to full equilibrium, where particle size and surface exposed no longer have an effect.

As seen from Table II, the $Ca:PO_4$ ratios undergo progressive increases with water and fluoride shaking. The $PO_4:2CO_3$ ratios undergo progressive decreases in the rat enamel and dentin with water and fluoride shaking, but decrease only with fluoride in the human teeth. The changes in $Ca:PO_4$ and $PO_4:2CO_3$ ratios are greater in the rat than in the human teeth. The residual $Ca:PO_4$ ratios of the untreated rat enamel and dentin are below 1.50, and higher than 1.50 for human enamel and dentin.

TABLE II

COMPOSITION OF TEETH BEFORE AND AFTER EQUILIBRATION WITH NaF AND H_2O AT pH OF 7.1
(The results represent molar ratios)

	TREATMENT								
	NONE			WATER			SODIUM FLUORIDE		
	$Ca:PO_4$	RESIDUAL $Ca:PO_4$	$PO_4:2CO_3$	$Ca:PO_4$	RESIDUAL $Ca:PO_4$	$PO_4:2CO_3$	$Ca:PO_4$	RESIDUAL $Ca:PO_4$	$PO_4:2CO_3$
Rat									
Enamel	1.47	1.38	5.21	1.80	1.68	4.40	2.11	1.97	3.71
Dentin	1.20	1.12	6.15	1.35	1.26	5.14	1.63	1.53	4.68
Human									
Enamel	1.79	1.72	5.15	1.89	1.79	5.24	2.02	1.91	4.55
Dentin	1.75	1.64	3.52	1.74	1.64	3.55	1.87	1.72	3.28

The changes in composition under the influence of fluoride are distinctly greater than those under the influence of water.

X-RAY DIFFRACTION PATTERNS OF TEETH BEFORE AND AFTER EQUILIBRATION WITH SODIUM FLUORIDE

The results of x-ray studies are summarized in Table III. Before heating, the predominant x-ray diffraction pattern is apatite for the enamel and dentin of both species. This was not altered by fluoride treatment. The existence of calcium fluoride as an independent solid could not be established from these studies since there was no significant change in those lines which represent calcium fluoride.

DISCUSSION

The enamel and dentin of both species give apatite patterns in spite of the wide variations of composition that were encountered (Table III). This confirms previous findings that apatites represent a series of isomorphous compounds whose composition may vary within wide limits.[7, 8] On ignition the enamel and dentin of the rat changed to β-$Ca_3(PO_4)_2$ while the human enamel and dentin continued to show the apatite pattern. The composition of rat enamel and dentin (Tables I and II) was analogous to the high phosphate bones which on heating gave β-$Ca_3(PO_4)_2$,[4, 9] the $Ca:PO_4$ ratio being less than 1.50. Materials giving β-$Ca_3(PO_4)_2$ patterns have been prepared by heating inorganic mixtures of $CaHPO_4$ and hydroxy apatite[4]; by igniting $Ca_3(PO_4)_2.2H_2O$ at 900°[10, 11]; by igniting bone after removing the carbonate with weak acids[12, 13] and by igniting high phosphate bones.[4]

TABLE III
PREDOMINANT X-RAY PATTERNS

	NO TREATMENT		NaF TREATMENT	
	BEFORE IGNITION	AFTER IGNITION	BEFORE IGNITION	AFTER IGNITION
Rat enamel	Apatite	β-$Ca_3(PO_4)_2$	Apatite	Apatite
Rat dentin	Apatite	β-$Ca_3(PO_4)_2$	Apatite	Apatite
Human enamel	Apatite	Apatite	Apatite	Apatite
Human dentin	Apatite	Apatite	Apatite	Apatite

The empirical composition of rat enamel and dentin may be represented by $[Ca_3(PO_4)_2]_n[CaHPO_4]_m[CaCO_3]$ just as it was for high phosphate bone.[9] The proportion of $CaHPO_4$ is higher in the dentin than in the enamel because of the lower Ca:PO_4 ratios. Taking into consideration studies on high phosphate bone,[4] the most likely reaction that took place on ignition would be as follows:

$$1.\ [Ca_3(PO_4)_2]_n[CaHPO_4]_2[CaCO_3] \xrightarrow{900^\circ} (n+1)\beta\text{-}Ca_3(PO_4)_2 + H_2O + CO_2$$

The composition of the rat teeth is such that on ignition β-$Ca_3(PO_4)_2$ is obtained.

Hodge, LeFevre, and Bale[14] found that when molar Ca:PO_4 ratios of commercial $Ca_3(PO_4)_2$ are less than 1.50, only the β-$Ca_3(PO_4)_2$ pattern is found on ignition. When these ratios are more than 1.60, the pattern, after ignition, is predominantly apatite. They gave weight ratios which were here converted to molar ratios.

This rule held true in the present investigations. Where the Ca:PO_4 ratio was less than 1.50, namely in the rat dentin and enamel, β-$Ca_3(PO_4)_2$ was found in the ignited specimens. Where the Ca:PO_4 ratio was more than 1.60, namely in the human teeth and in both the human and rat teeth after sodium fluoride treatment, apatite was found after ignition.

The composition of human teeth is not limited to that given in Table II. Bale, LeFevre, and Hodge[15] observed that human dentin occasionally gave the β-$Ca_3(PO_4)_2$ on ignition indicating (if we apply the rule in the preceding paragraph) that the Ca:PO_4 ratio was lower than that found in our teeth. Among human teeth that we analyzed, there was a molar tooth where the Ca:PO_4 ratio was 1.49 for the dentin and 1.81 for the enamel. This would represent the type of dentin which on heating would be likely to give β-$Ca_3(PO_4)_2$. The above authors concluded that the crystals of enamel, being larger than those of dentin, would not adsorb phosphate on their surfaces to any great extent and therefore would not give β-$Ca_3(PO_4)_2$. That enamel has larger crystals, both in human and rat teeth, is indicated by the fact, in our studies, that the enamels have sharper diffraction lines than the corresponding dentins. Nevertheless, rat enamel on heating did give β-$Ca_3(PO_4)_2$. If the large excess of phosphate in the rat enamel is not on the surface, it must be part of the lattice. That the composition that gives β-$Ca_3(PO_4)_2$ may not be exclusively the property of rat enamel may be indicated by a Ca:PO_4 ratio of 1.47 found for the enamel of a human premolar that we analyzed. The dentin for this particular tooth had a Ca:PO_4 ratio of 1.66. These scattered data indicate that in controlled studies, human teeth may show wide variations in composition analogous to those found in rat teeth.[1]

Enamel PO_4:$2CO_3$ ratios found in the upper incisors of rats[1] (and Table II) were higher in some of our groups and lower in others than those hitherto found in humans,[16, 17] (Table II and unpublished experiments). The lowest dentin PO_4:$2CO_3$ ratios found in the upper incisors of rats were of the order of the highest ratios found in human dentin. Therefore, from the hitherto available data, there appears to be at least one difference worth pointing out between human teeth and rat upper incisors. The PO_4:$2CO_3$ ratios of the enamels are higher in human and lower in the rat than the corresponding dentins[1, 16, 17] (Table II, and unpublished experiments).

The influence that diet may exert on composition by affecting the humoral and "local" factors has been discussed in our previous paper.[1] In the composition of rat teeth (as shown in Table II), there is another example of dietary influence. The Ca:PO_4 ratios of both enamel and dentin were lower than those previously found.[1] The differences between the stock diet used for these rats[5] and the experimental diets used previously[1] must play a part in the differences between compositions observed in these studies and earlier studies where a relationship was established between the PO_2:$2CO_3$ ratios of teeth and blood. It must be pointed out that the stock diet itself is not constant since the composition of each of the six ingredients may vary from batch to batch.[1, 5] Systematic investigation of not only the dietary Ca:P ratios but of the influence of amino acids, carbohydrates, lipids, and vitamins on composition must be undertaken to clearly understand the calcification of teeth.

Sodium fluoride is another example of a factor that influences the composition of teeth, at least in vitro. The changes (Tables I and II) were significantly greater than those obtained with water alone. The changes were in the direction one might expect. Calcium fluoride is an insoluble salt and as such the fluoride ion would tend to minimize the solubility of calcium. That the fluoride appeared to replace phosphate in preference to carbonate was unexpected. Logan and Taylor[18] and Dallemagne, Brasseur, and Melon[12, 13] showed that the carbonate in bone is removed, in preference to the phosphate, by weak acids. Whether we are dealing with fundamental differences between teeth and bone or merely pH effects requires further investigation.

On the basis of our x-ray studies, it cannot be determined whether fluorapatite or calcium fluoride was formed. It is difficult to determine the difference between fluorapatite and other apatites by means of the x-ray technics used here, due to the close similarity of lines.[19-21] Calcium fluoride, as such, was not detectable for the same reason.[21] The x-ray diffraction patterns of the fluoride-treated teeth gave apatite patterns (Table III). The fluoride-treated rat enamel and dentin gave apatite on ignition. In contrast to this, the incinerated rat enamel and dentin (without fluoride treatment) gave β-$Ca_3(PO_4)_2$. This difference can be explained by the Ca:PO_4 ratios which were well above 1.50 (Tables I-III). The following reactions explain the observed changes:

2. $[Ca_3(PO_4)_2]_n[CaHPO_4]_m[CaCO_3] + 2_mNaF \rightarrow [Ca_3(PO_4)_2]_n[CaF]_m[CaCO_3] + {}_mNa_2HPO_4$
(Apatite Pattern) (In Solution)

3. $[Ca_3(PO_4)_2]_n[CaF_2]_m[CaCO_3] \xrightarrow{900°} [Ca_3(PO_4)_2]_n[CaF_2]_m[CaCO_3]$
(Apatite Pattern) (Apatite Pattern)

If it is assumed that calcium fluoride is an independent solid, the reaction products would be: $[Ca_3(PO_4)_2]_{n-m}[CaCO_3] + [Ca_3(PO_4)_2]_m[CaF_2]_m$. Both give the apatite pattern on ignition.

The nature of these changes is such that a tooth which gives β-$Ca_3(PO_4)_2$ on ignition will give apatite on ignition after treatment with fluoride. A tooth which gives apatite on ignition before will continue to give apatite on ignition after fluoride treatment. This explains the results obtained for human teeth.

The sharper lines observed with enamel than with dentin is in agreement with previous observations[22] and is explained on the basis of the size of the crystals, those of enamel being larger than those of dentin. This fact may also be explained as being due to lesser amounts of organic matter in the enamel than in the dentin.[1, 16, 17, 23-25] The sharper diffraction lines obtained after heating agree with observations on bone.[12]

SUMMARY

1. The enamel and dentin of human and rat teeth, both before and after fluoride treatment, give apatite patterns in spite of the wide variations in composition that were encountered.

2. Where the $Ca:PO_4$ ratio was less than 1.50, namely in the rat dentin and enamel, β-$Ca_3(PO_4)_2$ was found in the ignited specimens. Where the $Ca:PO_4$ ratio was more than 1.60, namely in the human teeth and in both the rat and human teeth after sodium fluoride treatment, apatite was found after ignition.

3. Phosphate is lost in preference to carbonate under fluoride treatment at a pH of 7.1.

4. The $PO_4:2CO_3$ ratios of the enamels are higher in the human and lower in the rat than the corresponding dentins. (There is some evidence to indicate that more intensive investigation of human teeth may alter this conclusion.)

5. Enamel $PO_4:2CO_3$ ratios found in experimental animals were both higher and lower than those found hitherto in humans.

6. The $Ca:PO_4$ ratios of human enamel may be higher than, lower than, or equal to those of human dentin. The $Ca:PO_4$ ratios of rat enamel are always higher than those of rat dentin.

7. The $Ca:PO_4$ ratios of human dentin are usually higher than those of rat dentin. The range of $Ca:PO_4$ ratios of human enamel is smaller than that of rat enamel.

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PHOSPHATASE IN SALIVA

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ACCORDING to Miller's[1] classical chemicoparasitic theory, dental caries is caused by acids formed in the oral cavity. These acids, for example, lactic acid, are most likely degradation products of carbohydrate residues. In all probability they dissolve the enamel of the teeth in certain areas and expose the live dentin to bacterial action. The production of lactic acid may proceed by a scheme, such as the one proposed by Meyerhof,[2] for carbohydrate metabolism. This, and almost every other carbohydrate degradation, involves the formation of phosphate-containing intermediates, which require phosphatase for their hydrolysis.

Earlier workers have done some research on phosphatase in human saliva, but they came to different conclusions. Since they were interested more in other body fluids, organs, muscles, and bones, saliva was treated merely as one of the less important body fluids.

Demuth[3] stated that saliva did contain phosphatase, but in smaller quantities than bile. His hydrolysis experiments took as long as forty-eight hours. This length of time is certainly not typical of enzyme action and we suggest that he measured bacterial rather than enzyme action. Umeno[4] said that there was no phosphatase in saliva at all, though he found some in the salivary glands. Giri[5] reported that salivary phosphatase was identical with urinary phosphatase, basing this conclusion on a common optimum pH. Glock, Murray, and Pincus[6] found saliva to contain varying amounts of phosphatase. They also state that the phosphatase was not entirely due to epithelial cells but also to microorganisms. Rae[7] showed no relation between salivary phosphatase and dental caries. Smith[8] and Adamson[9] were of the opinion that the phosphatase came solely from desquamated epithelial cells.

These contradictory ideas and findings are due, in part, to the complexity of saliva and to variations in individual salivas. We shall, therefore, try to answer the following question: Is there a salivary phosphatase, or is it merely a phosphatase in saliva due to bacteria, cellular debris, or both?

EXPERIMENTAL

Phosphatatic activity was determined by the hydrolyzing action of saliva on an organic phosphate, disodium phenyl phosphate. The temperature at which the hydrolyses were carried out was 37° Centigrade. The final substrate concentration was 1/20 M throughout the experiments. The pH values, determined with a Beckman glass electrode pH meter, were varied to find the optimum

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pH. The buffers used were: Sodium acetate-acetic acid up to pH 6, Veronal-hydrochloric acid from pH 6-8, and Veronal-sodium carbonate from pH 8-10.

The extent of hydrolysis was determined by measuring colorimetrically the amount of free phenol liberated, using Folin and Ciocalteau's[10] method. As a check the amount of phosphate liberated was also determined by Ammon and Hinsberg's[11] adaptation of King's[12] colorimetric method. The color readings were made on a "Lumetron," a photoelectric colorimeter. The inorganic phosphate already present in saliva was measured by suitable controls.

Table I contains some of the average results of experiments conducted as follows: Into a numbered 50 ml. test tube 10 ml. buffer, 2 ml. saliva solution (1:1 mixture of saliva and distilled water) and 2 ml. substrate were pipetted, giving 14 ml. of a solution with the required pH and a 1/20 M concentration of disodium phenyl phosphate. For the control, the saliva was first placed into a boiling water bath for 20 minutes. The saliva used was a mixed unstimulated saliva ("resting") collected from forty-six students. The test tube was then placed in a rack mounted on a Warburg apparatus thermostated at 37° Centigrade. The figures given are the measurements at the end of one hour. The optimum pH for the phosphatase in saliva was found to be 5.

TABLE I
RELATION BETWEEN PH AND PHOSPHATATIC ACTIVITY OF WHOLE SALIVA

	MG. PHENOL LIBERATED PER 100 ML. SALIVA FROM 0.05 *M* DISODIUM PHENYL PHOSPHATE AT 37° C. IN ONE HOUR		
pH	FRESH SALIVA	BOILED SALIVA	DIFFERENCE
3.8	2.50	1.35	1.15
5.1	3.58	1.17	2.41
6.0	2.70	1.38	1.32
7.2	2.42	1.38	1.04
8.0	2.25	1.38	0.87
9.0	1.50	1.17	0.33

Table I shows the figures obtained when whole fresh saliva was used. The supernatant clear liquid from centrifuged saliva liberated only very small amounts of free phenol (0.002 mg. per ml. of saliva per first hour) when used in the preceding experiment. Even this trace of phosphatatic activity may have been due partly to the relatively slow rate of centrifuging (2200 r.p.m. for 20 minutes), since saliva filtered through a Seitz filter showed no phosphatatic activity whatsoever. The residue obtained on centrifuging whole saliva showed the same phosphatatic activity as whole saliva under the same conditions, that is, at pH 5.1 the residue per 100 ml. of saliva liberated in two hours 3.6 mg. phenol.

In order to be sure that during the experiments the activity was not lost, due to filtration or overlong exposure to air, a pork kidney extract was prepared from 0.3 kg. of fresh pork kidney. The kidney was placed in a Waring Blendor and completely minced up with some distilled water. This mixture was left standing overnight in the refrigerator and filtered next day through 2 cm. of filter-cel on top of a No. 42 Whatman filter paper. A total of 1 liter of solution was obtained of which 0.5 ml. were used for each experiment. A boiled sample

of the solution was used as a control. In the first hour slightly more than fifty times the amount of phenol was liberated by this 0.5 ml. sample than by a 1.0 ml. sample of whole saliva. A sample of this solution was passed through a Seitz filter and no decrease in activity was observed.

From these results it appeared that whatever small amount of phosphatase there is in saliva is not secreted by the glands, but gets into the saliva at a later stage. To support this theory an attempt was made to obtain cannulated human saliva. This work was done by Dr. J. Kreutzer of the Faculty of Dentistry at this university. The effort was unsuccessful because of the slow drainage of the glands under normal conditions. The only gland opening large enough to admit a canula was that of the parotid. From the others, sublingual and submaxillary glands, an attempt was made to remove the saliva with an eyedropper as soon as it appeared. The total amount thus obtained amounted to less than 0.5 milliliter. Further efforts will be made, however, to make such a saliva collection possible.

It was next thought advisable to investigate the microorganisms present in saliva, isolate them, and determine their phosphatase content. The mixed saliva used contained 100,000 *Lactobacillus acidophilus* per milliliter. It was found that the species used by a dairy was the same as one in human saliva. Therefore, a culture of *Lactobacillus acidophilus* in milk was obtained, plated out on tomato juice-agar plates (pH 5), and a pure culture started from the plate in carbohydrate broth. Broth prepared from lean beef with 2 per cent lactose added and buffered with sodium hydroxide and lactic acid was the most successful medium. Of the culture thus obtained, samples of 1 ml. were taken containing approximately 100,000 *Lactobacillus acidophilus* per milliliter. The control was again a boiled sample. In all other respects the experiments were exactly the same as those with saliva. In this set of experiments no measurable difference could be observed between the phosphatatic activity of the live and the boiled samples. If the phosphatase were intracellular, perhaps by breaking the cell walls of the bacillus a measurable amount of phosphatase could be obtained. As no bacterial mill was available, the "cracking" was attempted by alternate freezing and thawing of the culture. Hydrolysis experiments were conducted with the "cracked" *Lactobacillus acidophilus*, but still no appreciable amount of phosphatase could be measured.

DISCUSSION

If phosphatase can pass through a Seitz filter and filtered saliva shows no phosphatatic activity, it is reasonable to assume that saliva directly from the glands would contain no phosphatase either. That phosphatase can be filtered was ascertained by passing the pork kidney extract through the same filter and finding no appreciable decrease in activity.

The experiments with *Lactobacillus acidophilus* indicate that only a very small amount of the phosphatase comes from this bacillus. The remainder probably coming to a larger extent from cellular debris and to a smaller extent from other microorganisms.

Since *Lactobacillus acidophilus* constitutes an appreciable bulk of the oral flora, it may be assumed that microorganisms supply only a small amount of the phosphatase.

SUMMARY

1. Since filtered saliva showed no phosphatatic activity and since phosphatase passes unchanged through the filter, it may be assumed that saliva as secreted by the glands contains no phosphatase. The phrase "salivary phosphatase" should be changed therefore to the more exact "phosphatase in saliva."

2. Relative to the average population of *Lactobacillus acidophilus* in saliva of caries-susceptible (100,000 per ml.), only very small amounts of phosphatase are derived from these bacteria.

3. The small amount of phosphatase in saliva is probably derived from cellular debris and food residues, with a lesser contribution by microorganisms.

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THE HYDROGEN ION CONCENTRATION OF THE SALIVA

I. Factors Influencing CO_2 Content

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A REVIEW of the literature concerning the pH of saliva shows that there is no agreement among investigators as to the importance to be attached to CO_2 content as a factor influencing the salivary pH. It is generally accepted that saliva loses CO_2 during a long exposure to air, but many workers consider that during a short exposure the amount lost and the resulting pH change are negligible. Bloomfield and Huck (1920) found no significant pH change, whereas Starr (1922) found that the pH rose after exposure of saliva to air. Champion (1932) and Benetato and Oprisiu (1938) made pH determinations under a protective seal of oil, while Grossman and Brickman (1937) and Schmidt Nielsen (1946) used a closed system to prevent contact between saliva and air. Brown and Klotz (1935) and Eisenbrandt (1943) took no precautions against CO_2 loss.

Attention to the importance of salivary CO_2 content was directed by the work of Rapp (1946), which put forward evidence of the presence of the enzyme carbonic anhydrase in saliva. This enzyme catalyses the reaction $H_2CO_3 \rightleftharpoons H_2O + CO_2$ and will hasten the evolution of CO_2 in the presence of a favorable pressure gradient. Under the influence of this enzyme, the amount of CO_2 lost during a short exposure to air might be sufficient to cause a marked pH change.

Experiments were planned to determine the conditions of CO_2 loss from saliva. The objects of these experiments were: 1. to expose saliva to air for varying periods of time and measure the CO_2 content before and after exposure, and 2. to determine the effect on CO_2 evolution of amounts of sulfanilamide sufficient to inhibit carbonic anhydrase. Experiments to demonstrate the presence of carbonic anhydrase in saliva as described by Rapp were repeated.

Although Keilin and Mann (1940) showed that the purified enzyme was inhibited by concentrations of sulfanilamide as low as 2×10^{-6} M, Davenport (1945) and Anderson and Thomson (1948) have demonstrated that in a blood solution not less than 50 mg./100 ml. is required. It was therefore decided to use a concentration of at least 50 mg. of sulfanilamide per 100 ml. of saliva. Tables 1 and 2 show the results of the preliminary experiments, and it will be seen that considerable difference in initial CO_2 content exists between stimulated and unstimulated saliva. In view of these differences, it was thought desirable to test CO_2 evolution on the two types of saliva.

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METHOD

Stimulated saliva was obtained by chewing paraffin wax with the lips firmly sealed; unstimulated saliva was that which collected in the mouth in the absence of any specific stimulus to secretion. When a large volume had collected, it was deposited into a beaker under liquid paraffin. From this it was immediately transferred to a graduated burette over mercury. The burette had a tap and delivery tube sealed onto its upper end, and was connected at its lower end to a mercury manometer. The burette and delivery tube first were filled with mercury; then with the tip of the delivery tube in the saliva under paraffin, the mercury reservoir was lowered and saliva drawn into the burette. A little mercury following the saliva served as a seal. Measured volumes of saliva could be drawn from the burette when required.

Saliva samples of 3 ml. volume were delivered into small beakers and allowed to remain in contact with air for varying periods of time. The times chosen were one, two, four, and six minutes. The exposure was terminated by pouring a thick layer of liquid paraffin into the beaker. The saliva was quickly transferred to modified Hempel pipettes in which measured volumes could be stored between columns of mercury until required for analysis. CO_2 content was estimated with the Van Slyke volumetric apparatus. Three solutions of sodium bicarbonate were treated in the same manner so that CO_2 evolution could be compared with that from saliva.

RESULTS

The results are shown in Tables I, II, and III. The CO_2 content of every specimen after different exposure times are given in the tables, and the mean CO_2 losses are shown at the foot of each column. Student's distribution of "t" was used to test the significance of these values, and all the mean CO_2 losses from unstimulated saliva were found significant, taking a probability of 0.05 as the level of significance. None of the CO_2 losses from stimulated saliva was found to reach the required level of significance. The CO_2 contents of the three bicarbonate solutions are shown in Table III. The concentrations of the original solutions were chosen to resemble the two types of saliva, and there has obviously been no significant CO_2 loss even after six minutes exposure. Thus it appears

TABLE I

SHOWING THE EFFECT OF EXPOSING UNSTIMULATED SALIVA TO AIR

CO_2 VOLS. % NONEXPOSED CONTROL	EXPOSURE TIMES			
	1 MIN.	2 MIN.	4 MIN.	6 MIN.
17.3	—	15.1	14.5	13.8
15.4	14.4	13.4	12.6	12.5
11.5	11.1	11.1	11.0	10.9
17.4	16.2	15.8	14.3	13.5
13.5	13.2	13.3	12.2	11.5
Mean CO_2 loss from control	0.73	1.28	2.10	2.60
"t"*	3.278	3.101	4.22	4.45
P†	<0.05	<0.05	<0.02	<0.02

*The distribution of "t" after the method of Student.
†Probability.

TABLE II
SHOWING THE EFFECT OF EXPOSING STIMULATED SALIVA TO AIR

CO_2 VOLS. % NONEXPOSED CONTROL	EXPOSURE TIMES			
	1 MIN.	2 MIN.	4 MIN.	6 MIN.
47.4	47.1	47.4	46.5	43.3
53.5	51.7	52.9	53.8	54.6
50.6	50.0	49.8	49.4	49.6
49.1	48.8	47.7	48.0	47.4
50.4	50.2	50.6	50.0	48.1
Mean CO_2 loss from control	0.64	0.64	0.66	1.60
"t"	2.15	1.811	2.39	1.89
P	>0.05	>0.1	>0.05	>0.1

TABLE III
SHOWING THE EFFECT OF EXPOSING SODIUM BICARBONATE SOLUTION TO AIR

CO_2 VOLS. % NONEXPOSED CONTROL	EXPOSURE TIMES			
	1 MIN.	2 MIN.	4 MIN.	6 MIN.
15.7	16.0	16.7	16.3	15.9
16.8	—	16.1	15.8	16.6
46.9	—	48.0	46.2	47.5
Mean difference	+0.3	+0.8	−0.3	+0.3

that stimulated saliva behaves like a bicarbonate solution when exposed to air, whereas unstimulated saliva loses CO_2 more rapidly.

There are two possible explanations for this difference of behavior. Schmidt Nielsen (1946) and others have shown that the pH of resting saliva is lower than that of stimulated saliva, and this pH difference might account for the different rates of CO_2 loss. The second possibility is that carbonic anhydrase might be present in higher concentration or more active state in unstimulated than stimulated saliva. A second series of experiments was planned to test this possibility.

About 15 mg. of sulfanilamide was mixed with 20 ml. of unstimulated saliva under paraffin before transferring the saliva to the graduated burette already described. CO_2 evolution was tested as before. Sulfanilamide concentration was estimated by the method of Rose and Bevan (1944). The results of this series of experiments are shown in Table IV.

TABLE IV
SHOWING THE EFFECT OF EXPOSING UNSTIMULATED SALIVA CONTAINING SULFANILAMIDE TO AIR

CO_2 VOLS. % NONEXPOSED CONTROL	EXPOSURE TIMES				SULFANILAMIDE CONCENTRATION MG./100 C.C.
	1 MIN.	2 MIN.	4 MIN.	6 MIN.	
17.4	16.9	16.4	15.8	15.9	77.4
18.8	18.0	17.3	16.6	15.3	86.0
13.1	12.7	11.6	11.6	10.7	84.5
14.9	13.8	13.3	12.4	12.1	89.2
15.0	14.7	14.3	14.8	13.1	—
Mean CO_2 loss from control	0.62	1.26	1.60	2.42	
"t"	4.239	7.204	4.03	6.9	
P	<0.02	<0.01	<0.02	<0.01	

The mean CO_2 losses and value of "t" are given below each column, and the sulfanilamide concentrations are shown in the last column of the table. All the differences reached the level of significance required. The mean CO_2 losses in the presence of sulfanilamide bear close resemblance to those obtained without sulfanilamide. In order to determine whether there was in fact any difference, the values were tested by the application of the "t" test advocated by Yule and Kendall (1940) for the comparison of two means. No significant difference between the means was found.

DISCUSSION

The results of the sulfanilamide experiments strongly suggest that the different rates of CO_2 evolution from stimulated and unstimulated saliva cannot be due to the presence of carbonic anhydrase in unstimulated saliva. The CO_2 content of stimulated saliva after six minutes' exposure does not differ significantly from the unexposed control, and in this way it behaves like a bicarbonate solution of comparable concentration. This observation made it necessary to repeat Rapp's experiments on stimulated saliva in which the presence of carbonic anhydrase was demonstrated. The evolution of CO_2 in a modified Roughton apparatus from a bicarbonate solution at pH 6.8 was measured in the presence or absence of 1 ml. of stimulated saliva, which had been dialyzed for 18 hours as described by Rapp. No increase in the rate of CO_2 evolution was recorded in the presence of the dialyzed saliva, and the author was therefore unable to repeat the results found by Rapp.

In view of the experiments with sulfanilamide on unstimulated saliva and the experiments with the Roughton apparatus on stimulated saliva, the conclusion is drawn that the different rates of CO_2 evolution from saliva are not due to the presence of an enzyme.

The rise in salivary pH coupled with increased rate of flow will be reported in a succeeding paper.

SUMMARY

1. The CO_2 content of saliva after periods of exposure to air has been measured.

2. The CO_2 loss from unstimulated saliva after an exposure of only one minute is statistically significant, whereas there is no significant CO_2 loss from stimulated saliva even after six minutes' exposure.

3. The addition of sulfanilamide does not effect the CO_2 loss from unstimulated saliva.

4. The presence of carbonic anhydrase in stimulated saliva has not been confirmed.

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OBSERVATIONS ON EXPERIMENTAL DENTAL CARIES

II. The Effect of Two Similar Diets Having the Same Initial pH and the Same Growth-Promoting Action

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THE findings on the relationship between the nutritional value of a diet and the incidence of caries are very contradictory. Based upon observations in human beings, Mellanby, Pattison, and Proud,[1] Howe, White, and Elliot,[2] and Boyd and his co-workers[3-8] have concluded that the high nutritional value of the diet is of a primary importance for the prevention and control of caries. On the other hand, the findings of Cohen,[9] studying normal and diabetic children, as well as those of Kniesner, Mann, and Spies,[10] and Mann, Dreizen, Spies, and Hunt,[11] comparing the incidence of caries in well and malnourished subjects, are in contradiction with the results reported by Mellanby, Howe, and Boyd. In laboratory animals, McCollum, Simmonds, Kinney, and Grieves[12] found that in albino rats a well-balanced diet offered protection against caries as compared with various deficient rations. On the other hand, Keyes,[13] in hamsters, and Schweigert and his co-workers,[14, 15, 16] in cotton rats, have found that the high nutritional value of certain diets, as judged by their growth-promoting action, proved incapable of offering protection against caries. On the contrary, the animals fed rations which produced higher growth rates exhibited higher caries activity.

Schweigert and his colleagues[15, 16] have observed significant reduction of caries incidence through the partial substitution of the dietary sucrose by lard, casein, or by both. These workers found, however, that the additional fat and protein could not account for the decrease of caries incidence by a mere reduction of the sucrose content of the ration.

Taking into consideration the works just reviewed, and certain preliminary observations of the authors, we have tested the effect of two similar diets having the same growth-promoting action and the same initial pH. The two rations were outlined in such a way as to include in both of them 70 per cent of their content as ingredients of identical nature, mostly carbohydrates. The essential differences between the remaining 30 per cent of the two diets consisted in the kinds and quantities of protein, fat, and carbohydrates.

EXPERIMENTAL

Thirty Syrian hamsters, 23 days old, from litters of a stock colony maintained on a diet of Purina Laboratory Chow* and raw milk, were litter-mate

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*From Ralston Purina Company, St. Louis, Mo., U. S. A.

distributed into 2 groups of 15 animals each (8 males and 7 females). The animals were set in screen bottom cages without bedding, and reared for 100 days on the 2 diets shown in Table I. The rations, as well as distilled water, were available ad libitum, and the animals were weighed every week This experiment was carried out between the months of November and February.

TABLE I
COMPONENTS OF THE TWO DIETS, EXPRESSED IN PERCENTAGE OF GRAMS

INGREDIENTS	DIETS	
	NO. 1	NO. 2
Ground yellow corn	40	40
Sucrose	20	20
Cornstarch	5	5
Potato starch	5	5
Water-washed casein	16	
Ether-extracted yeast	8	5
Salt mixture*	3	
Lard	3	
α-Tocopherol acetate	0.010	
Synkayvite	0.001	
Powdered whole milk		19
Alfalfa meal		5
Sodium chloride		1

*The salt mixture was McCollum's Salt Mixture No. 185, supplemented with 13.5 mg. KI, 139 mg. $CuSO_4$, $5H_2O$, and 556 mg. $MnSO_4$, $4H_2O$ per 100 grams.

The particle size of the ground corn, casein, and yeast was distributed as follows. Corn: 100 per cent through a 12.5-mesh screen, 63 per cent through a 25-mesh screen, and 31 per cent through a 50-mesh screen. Yeast: 100 per cent through a 12.5-mesh screen, 82 per cent through a 25-mesh screen, and 4 per cent through a 50-mesh screen. Solutions of the diets were made with distilled water and the pH of them was determined with a glass-electrode potentiometer. The ration fed to Group 1 had a pH of 5.51, and that given to Group 2 had a pH of 5.55.

At the end of the experimental period the animals were sacrificed and autopsy was performed on all of them. After fixation in 10 per cent formalin the jaws were washed and dried. The molars were examined under a low-power dissecting microscope (x 15), and the gross carious lesions were scored using Keyes' method.[17]

RESULTS

The animals from both groups exhibited the same healthy appearance. Likewise, the growth rate of both groups was essentially the same (see Fig. 1). The autopsy did not reveal gross changes other than those encountered in the oral cavity.

Gingival atrophy and alveolar resorption were found in most of the animals of Group 1. These changes, though present in some animals of Group 2, were less marked. A yellow-brown stain in the enamel cuticle of the molars, unrelated to caries activity, was generally present in both groups.

TABLE II
CARIES ACTIVITY IN THE TWO GROUPS

	GROUP 1			GROUP 2		
Number of experimental animals	8♂♂	7♀♀	8♂♂ + 7♀♀	8♂♂	7♀♀	8♂♂ + 7♀♀
Number of animals affected	8	7	15	8	7	15
Number of carious molars	43	33	76	62	62	124
Average number of carious molars	5.3	4.7	5.0	7.7	8.8	8.2
Number of carious lesions	46	36	82	90	90	180
Average number of carious lesions	5.7	5.1	5.4	11.2	12.8	12
Average scores of carious lesions	1.2	0.9	1.1	2.5	2.4	2.4

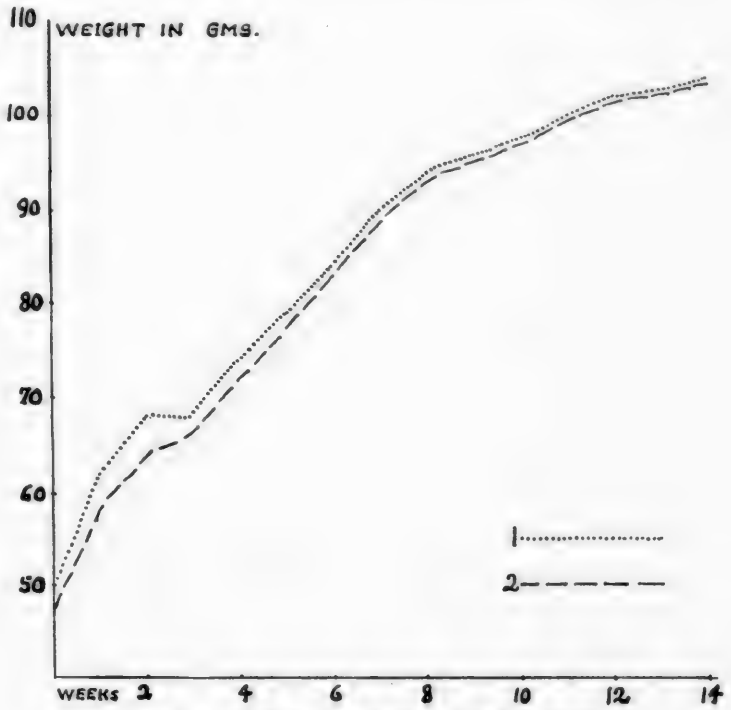

Fig. 1.—Average weight curves of the two groups.

Most of the carious lesions were encountered in the occlusal fossae. The grooves and the smooth surfaces were comparatively less affected. Comparing the caries activity of the two groups (Table II) higher incidence and extent of lesions are found in Group 2. The analysis of the differences shows that they are of a high statistical significance. Table II also shows that although in Group 1 the males exhibited higher caries activity than the females, this was not the case in Group 2. Therefore, in relation to caries activity, no sex difference was consistently found.

DISCUSSION

Both diets induced the same growth rate in spite of the fact that the ration fed to Group 1 had a considerable higher total protein content. This fact indicates the possibility that the powdered milk and/or the alfalfa of the ration fed to Group 2 contained a factor or factors which compensated the lower growth rate to be expected from the lower protein content of Diet 2.

Correlating the nutritional status of the two groups, as judged by their growth rate and health appearance, with their caries activity, it can be seen that in spite of exhibiting the same nutritional status the two groups had statistically significant differences in caries activity. This fact shows that the cariogenic power of two diets may be very different in spite of their having the same growth-promoting action.

The two diets had essentially the same initial pH, concomitant with a different capacity to induce caries. This shows that even if the initial pH of two rations is the same, the degradation products of their components once in the oral cavity can induce very different degrees of caries activity. It should not be impossible to outline two diets which, in spite of their having markedly different pH's, could have essentially the same cariogenic power.

Since 70 per cent of the content of both diets was supplied as identical ingredients, mostly carbohydrates, it is mainly the differences between the remaining 30 per cent of the two diets which should be taken into consideration. In Diet 1 the carbohydrates were almost totally excluded from this 30 per cent, whereas in Diet 2 the 19 per cent of powdered milk supplied an additional 7.42 per cent of carbohydrate in the form of lactose. This increase in a fermentable carbohydrate could be one of the factors responsible for the higher caries activity found in Group 2. In Diet 1 the fat content was mainly 3 per cent lard, and in Ration 2 was 5.37 per cent butterfat, supplied by the powdered milk. Although Diet 2 had a slightly higher fat content, the lard of Ration 1 supplied this diet with a physically free fat, a fact which from the dental viewpoint is more likely to have offered some protection against caries to Group 1. In this connection, we have observed that in hamsters lard actually offers a partial protection against caries.[18]

A third factor, perhaps not the least important, are the quantitative differences of protein in the two diets. Diet 1 contained 23.60 per cent of protein, supplied by the casein, yeast, and corn. Diet 2 contained only 12.52 per cent, supplied by the powdered milk, corn, yeast, and alfalfa. Since Diet 1 induced a lower caries activity, concomitant with higher periodontic disturbances, it is possible that the differences in protein content of the two diets may have played an important role in the causation of such an inversal relationship between the incidence of caries and of periodontal lesions in both groups. This possibility would agree with the results of the investigators who have found that some amino acids and certain products of protein putrefaction may decrease the incidence of caries at the same time that they may produce marked periodontal disturbances.[19, 24]

An analysis of the granular components of the two diets shows that in 70 per cent of their content the amounts of corn, with 31 per cent of its granules passing through a 50-mesh screen, were equal in both diets. In the remaining 30 per cent of the two diets the amounts of yeast, with its very large particle size, were not very different. On the other hand, 16 per cent of the content of Diet 1 was supplied by the casein, with 55 per cent of its granules passing through a 50-mesh screen. Taking into consideration the granules that passed a 50-mesh screen (31 per cent of the corn and 55 per cent of the casein) had a size more suitable for retention and impaction, it is possible that a good amount of casein may have taken the place of the corn in the sites suitable for retention and impaction, thus lowering the caries activity of Group 1. On the other hand, in Diet 2 the corn did not have free protein as a competitor. On the contrary, this diet contained 19 per cent of powdered milk, which supplied 5.32 per cent of protein, but at the same time supplied together 7.42 per cent of lactose. The presence of this additional amount of fermentable carbohydrate may have been another factor which contributed to the higher caries activity of Group 2.

Table II shows significant differences in the caries activity of the two groups. On the other hand, it also shows that the incidence and extent of caries were not high, even for Group 2. We have reported a similar observation in hamsters reared on purified diets, also coming from parents which belonged to a colony maintained on Purina Laboratory Chow and raw milk for a good number of generations.[18] Since the Purina Laboratory Chow has a high fluorine content, 37.9 p.p.m.,[25] it might be possible that this substance could, at least partially, be responsible for the low caries susceptibility of the first generation of these animals raised on purified diets. In this connection, Sognnaes has made some preliminary suggestions on the possible role of food purification in the etiology of caries.[26]

SUMMARY

Two groups of hamsters, 23 days old, were reared for 100 days on two similar diets having the same initial pH and the same growth-promoting action. The two rations had in common that 70 per cent of their content consisted of identical ingredients, mostly carbohydrates. In the remaining 30 per cent, the two diets differed quantitatively and qualitatively in certain of their components, as well as in their physical character.

Although both groups exhibited the same healthy appearance and the same growth rate, Group 1 had an incidence and extent of caries significantly lower than Group 2. Some of the factors which may have accounted for these differences in caries have been discussed.

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DETERMINATION OF OXYGEN CONSUMPTION IN NORMAL AND INFLAMED HUMAN GINGIVA USING THE WARBURG MANOMETRIC TECHNIC

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THE demonstrated value of the Warburg manometric technic for the study of the normal oxygen consumption of numerous body tissues[1, 2] and for the evaluation of metabolic changes in various phases of inflammation[3, 4, 5] suggested its possible applicability in the field of gingival disease.

It was felt that by indicating alterations in the metabolic activity of the gingivae, both on a physiological and pathological level, this method might point to relationships between gingival disease and various causative factors which have thus far evaded detection by the usual clinical and histological methods. Because of the frequency with which gingival inflammation and associated proliferative and degenerative changes occur in gingival disease, it was felt that information regarding their effect upon gingival metabolism as it is reflected in oxygen consumption would be particularly pertinent.

In contrast with its widespread use for the study of other tissues of the body, the literature contains only a single exploratory report[6] in which the Warburg manometric technic has been used for the study of oxygen consumption of human gingiva. A correlated histological and manometric study was therefore undertaken in an effort to determine first, whether the oxygen consumption of human gingiva could be measured by the Warburg technic; and second, to determine the relationship, if any, between oxygen consumption and the microscopic inflammatory changes commonly seen in gingival disease.

EXPERIMENTAL METHOD

The Warburg manometric technic[7] for measuring oxygen consumption is based upon the principle that if temperature and volume of a gas are maintained constant, any changes in the amount of gas in a given volume will be measurable by changes in pressure. If tissue is placed in a flask containing a measured amount of known fluid and connected with a graduated manometer so that the volume of gas between the surface of the fluid in the flask and the liquid in the manometer is maintained constant, then any alteration in the quantity of gas within this constant volume will produce changes in pressure which are measurable in millimeters of manometric fluid.

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Differences in the levels of manometric fluid in millimeters can subsequently be converted into cubic millimeters of gas either consumed or produced by the tissue in the flask during a given experimental period. Oxygen consumption, expressed as QO_2 value, indicates c mm. of oxygen taken up per mg. of dry weight of tissue per hour. In this report, the oxygen consumption of human gingival tissue will be expressed in QO_2 values.

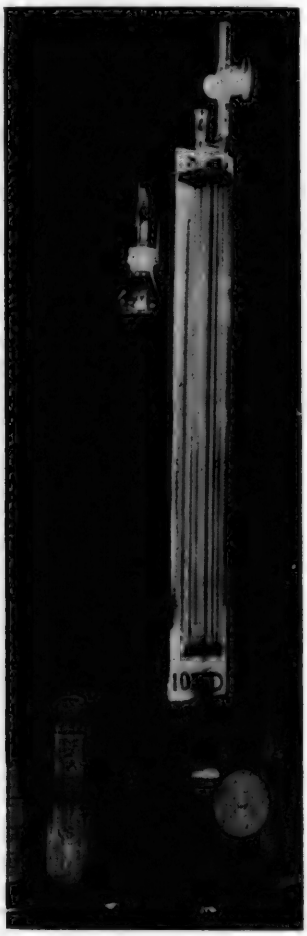

Fig. 1.—The Warburg constant volume manometer showing flask and attached graduated manometer.

Eighty-six specimens of gingival tissue from forty-six patients were used in this experiment. The tissue was removed with a scalpel or a scissors and placed immediately in a vial containing modified Ringer's solution (MPBR), buffered with phosphate (pH 7.5). A portion from each specimen was set

aside for microscopic study. All the operations were performed under local anesthesia (Novocain, 2 per cent; Adrenalin, 1-20,000).

The specimens consisted of marginal gingiva (with the exception of five specimens of oral mucosa over edentulous alveolar ridges) which ranged from 5 to 40 mm. in length; 3 to 8 mm. in width; and 3 to 5 mm. in thickness. Represented in the specimens were normal gingivae and gingivae with varying degrees of chronic inflammation and enlargement taken from patients 13 to 60 years of age.

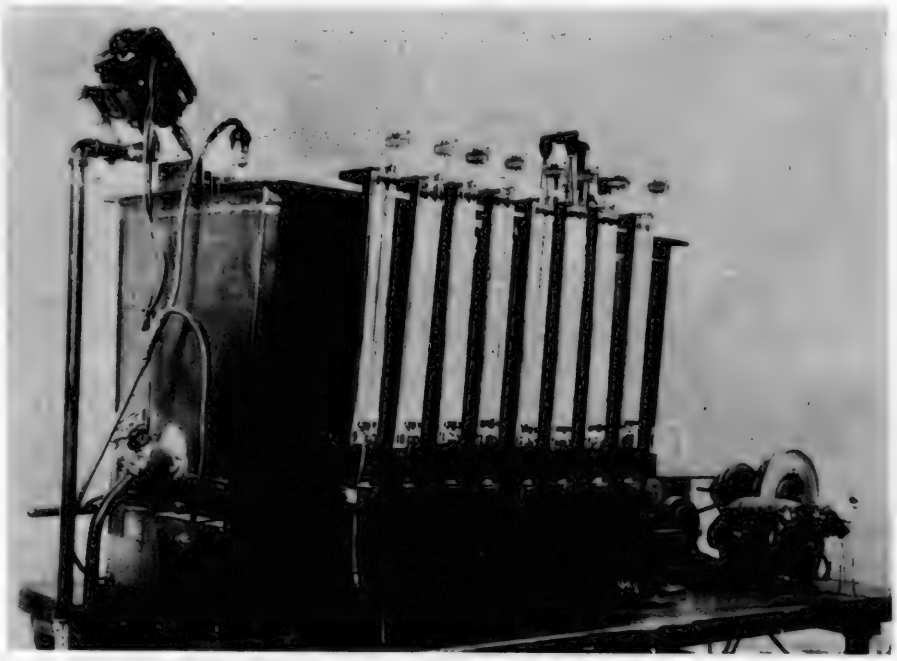

Fig. 2.—Warburg constant temperature water bath with manometers in place.

After collection, the gingival tissue was cut into small pieces with a razor blade with the intent of obtaining sections approximately 0.5 mm. to 1.0 mm. thick, and was placed in a flask containing 3 c.c. of phosphate-buffered Ringer's solution, pH 7.5 (Fig. 1). The center well of each flask contained a roll of filter paper saturated with .10 c.c. of 20 per cent NaOH for the absorption of CO_2 produced by the tissue respiration. The flasks were filled with oxygen for fifteen seconds, attached to a manometer, and placed in a water bath for equilibration at 37.5° C. for fifteen minutes. Stop cocks were then closed and zero readings were made. The intervals between the time of excision of the gingivae and the end of equilibration at constant temperature varied from thirty minutes to seventy-five minutes.

The flask and manometer were shaken in the water bath at 37.5° C. for one hour (Fig. 2), at the end of which time final manometric readings were made and the pH of the media determined. The tissue was then filtered through weighed crucibles and thoroughly washed. The crucibles and tissues

Fig. 3.—Normal (Group 1). Normal gingiva with stratified squamous epithelial surface and rete pegs extending into the underlying connective tissue. A few scattered inflammatory cells are seen in the densely collagenous connective stroma. Hematoxylin and eosin. Orig. mag. 25×.

Fig. 4.—Slight inflammation and proliferation (Group 2). Gingiva showing parakeratosis and slight vacuolar degeneration of the stratified squamous epithelium and accumulations of inflammatory cells in the underlying connective tissue. Hematoxylin and eosin. Orig. mag. 35×.

were kept in an oven overnight at 90° C. and the dry weight of the tissue recorded in the morning. The samples in each crucible varied in weight from 3.3 to 81.1 mg., but the majority ranged from five to ten milligrams.

RESULTS

A. Gingival Biopsies:

The gingival specimens could be divided into the following four groups on a histopathological basis.

1. Normal—specimens which presented a minimal inflammatory proliferative or degenerative change (Fig. 3).

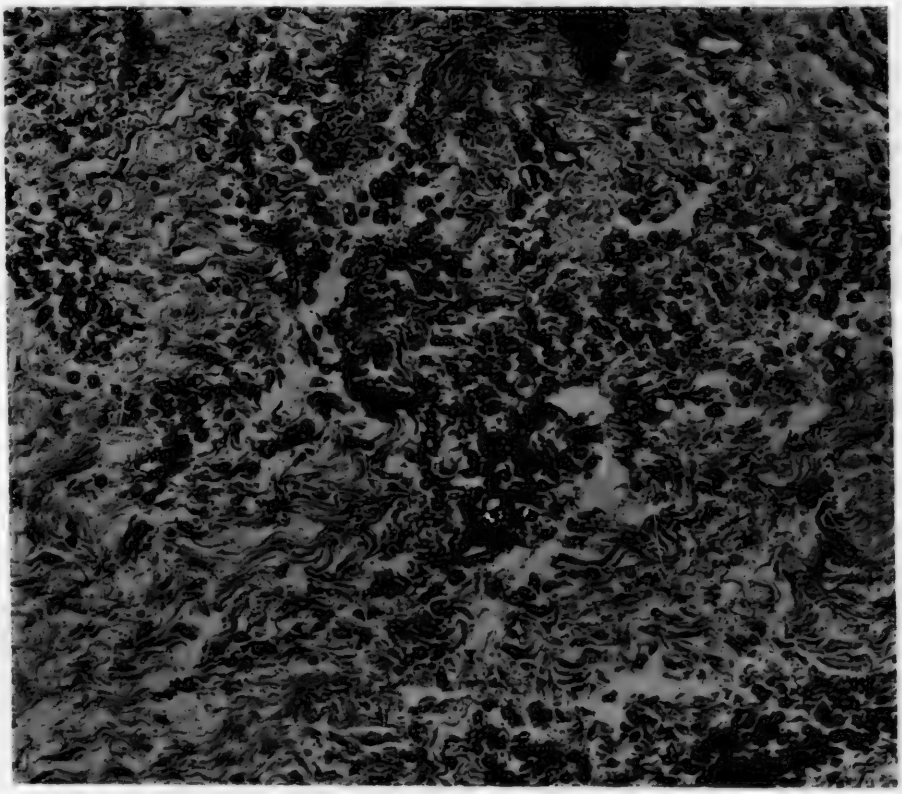

Fig. 5.—Detail of Fig. 4 (Group 2). Showing accumulation of lymphocytes and plasma cells with edema and degeneration of the surrounding collagen. Orig. mag. 400×.

2. Slight inflammation and proliferation—specimens which presented slight chronic inflammatory infiltration, and/or proliferation of epithelium and connective tissue (Figs. 4 and 5).

3. Marked inflammation and proliferation—specimens which presented marked chronic inflammmatory infiltration and/or proliferation of epithelium and connective tissue and new blood vesssels (Figs. 6-10).

Fig. 6.

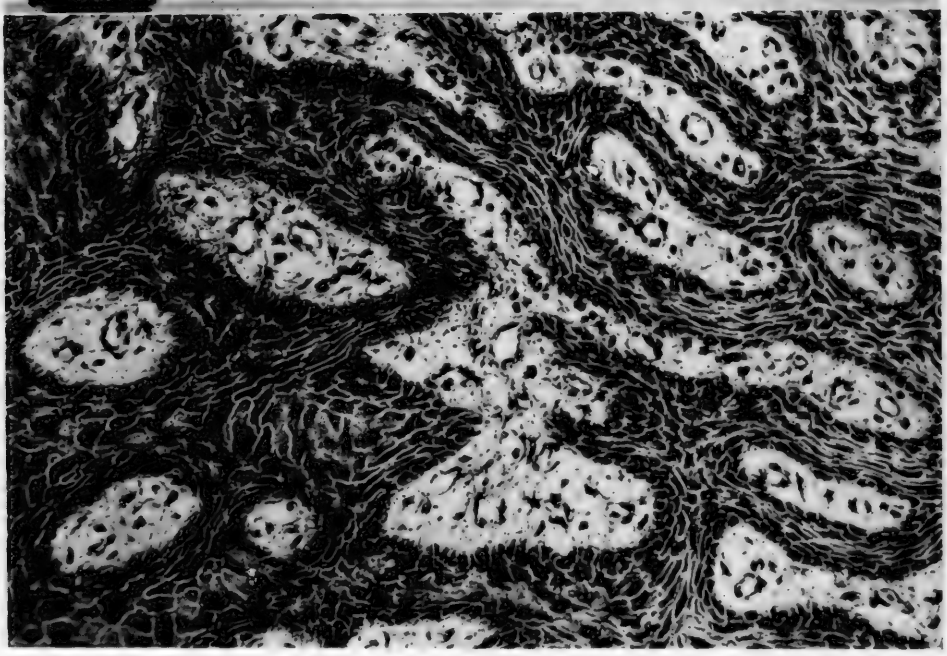

Fig. 7.

Fig. 6.—Marked inflammation and proliferation (Group 3). Tab of enlarged gingiva showing hyperplasia of the stratified squamous epithelium and inflammation in the connective tissue core. Marked degeneration of both the epithelium and connective tissue is seen in the gingival surface which approximated the teeth (lower right). Hematoxylin and eosin. Orig. mag. 25×.

Fig. 7.—Detail of Fig. 6 (Group 3). Showing connective tissue enclosed within strands of hyperplastic epithelium. Note numerous young capillaries in the connective tissue. Hematoxylin and eosin. Orig. mag. 400×.

Fig. 8.

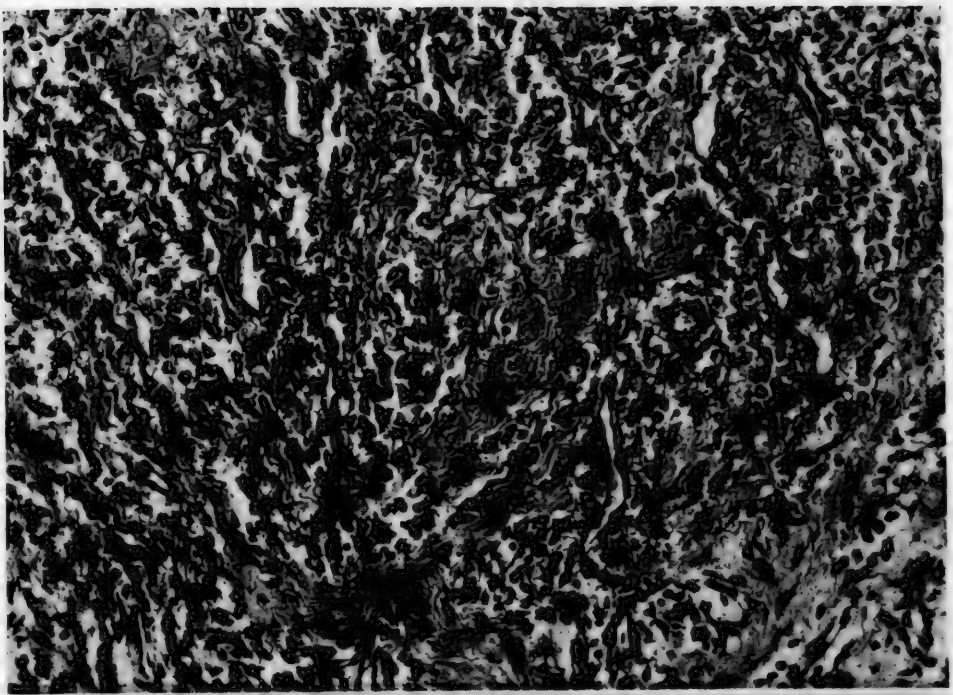

Fig. 9.

Fig. 8.—Detail of Fig. 6 (Group 3). Showing dense inflammatory infiltration and interspersed collagen fibres. Note degeneration of the collagen. Hematoxylin and eosin. Orig. mag. 200×.

Fig. 9.—Marked inflammation and proliferation (Group 3). Enlarged gingiva showing epithelial hyperplasia and marked inflammation in the underlying connective tissue. Note necrotic area at gingival margin (extreme right). Hematoxylin and eosin. Orig. mag. 25×.

4. Marked degeneration—specimens in which degeneration and necrosis were the only or predominant findings (Figs. 11, 12).

In most instances, the microscopic appearance of the individual specimens varied in the different areas of the prepared section. The above grouping is in accordance with the predominant changes.

B. Oxygen Consumption Values (QO_2):

The distribution of QO_2 values obtained for all the forty-six patients is shown in Table I.

TABLE I

NO. PATIENTS	% PATIENTS	QO_2 RANGE
8	17.4	0.2 to 0.9
17	37.0	1.0 to 1.9
15	32.6	2.0 to 2.9
6	13.0	3.0 to 4.4

C. Correlation of Microscopic Changes and Oxygen Consumption Values:

The individual QO_2 values grouped according to histopathological appearance is presented in Table II.

TABLE II

GROUP 1: NORMAL		GROUP 2: SLIGHT INFLAMMATION AND PROLIFERATION		GROUP 3: MARKED INFLAMMATION AND PROLIFERATION		GROUP 4: MARKED DEGENERATION	
CASE NO.	QO_2	CASE NO.	QO_2	CASE NO.	QO_2	CASE NO.	QO_2
4	2.8	8	1.3	3	1.1	19	1.5
9	3.0	30	1.7	7	2.9	22	0.7
10	0.7	39	1.7	36	1.1	33	0.5
38	1.2	24	2.2	27	2.6	34	1.2
40	0.9	25	1.9	32	2.9	16	1.4
41	2.4	28	2.0	43	1.4		
42	0.2	31	2.4	50	3.2		
45	1.5	46	1.1	56	3.7		
		51	1.7	61	2.2		
		57	2.2	55	4.4		
				59	3.6		
Average and Standard Deviation	$1.6 \pm .37$		$1.8 \pm .13$		$2.6 \pm .33$		$1.1 \pm .20$

Correlation of the QO_2 values with the underlying microscopic gingival changes represented in Table II reveals the following:

1. The average oxygen consumption for normal gingivae (Group 1) was $1.6 \pm .37$.

Fig. 10.—Detail of Fig. 9 (Group 3). Showing dense infiltration of lymphocytes and plasma cells and marked degeneration of collagen. Hematoxylin and eosin. Orig. mag. 200×.

Fig. 11.—Marked degeneration (Group 4). Gingival tab showing epithelial hyperplasia and generalized edema. Hematoxylin and eosin. Orig. mag. 90×.

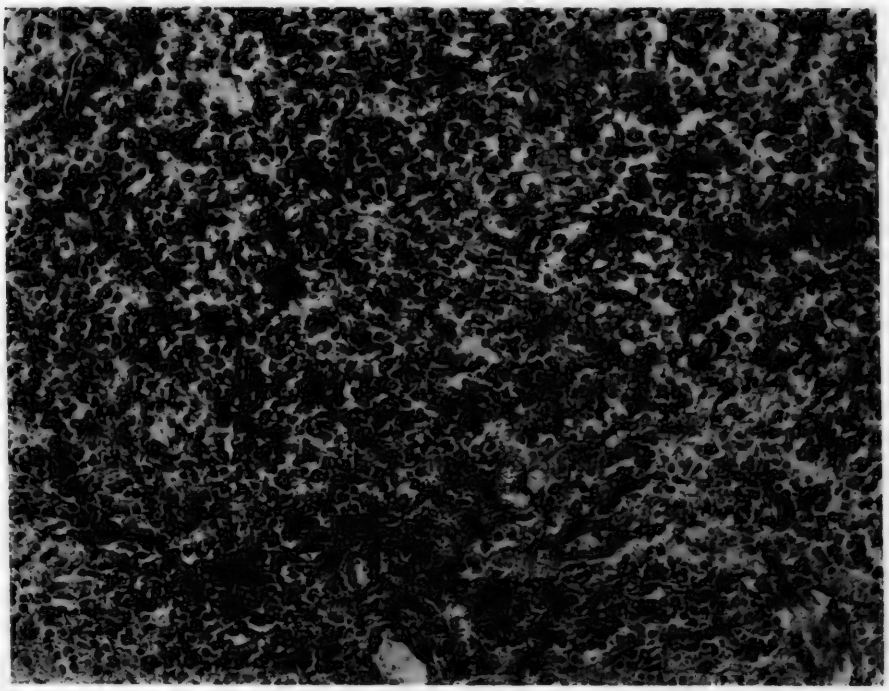

Fig. 10.

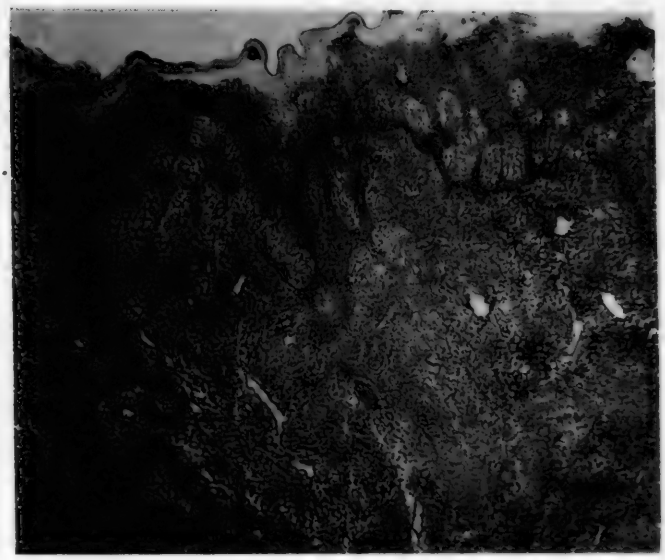

Fig. 11.

(See opposite page for legends.)

2. Oxygen consumption was essentially unaltered in gingivae with slight inflammatory and/or proliferative changes (Group 2).

3. There was a tendency toward increased oxygen consumption in gingivae with marked chronic inflammation and proliferation of connective tissue and epithelium (Group 3).

4. There was a tendency toward decreased oxygen consumption in gingivae which were essentially degenerated and necrotic and contained few, if any, viable tissue elements (Group 4).

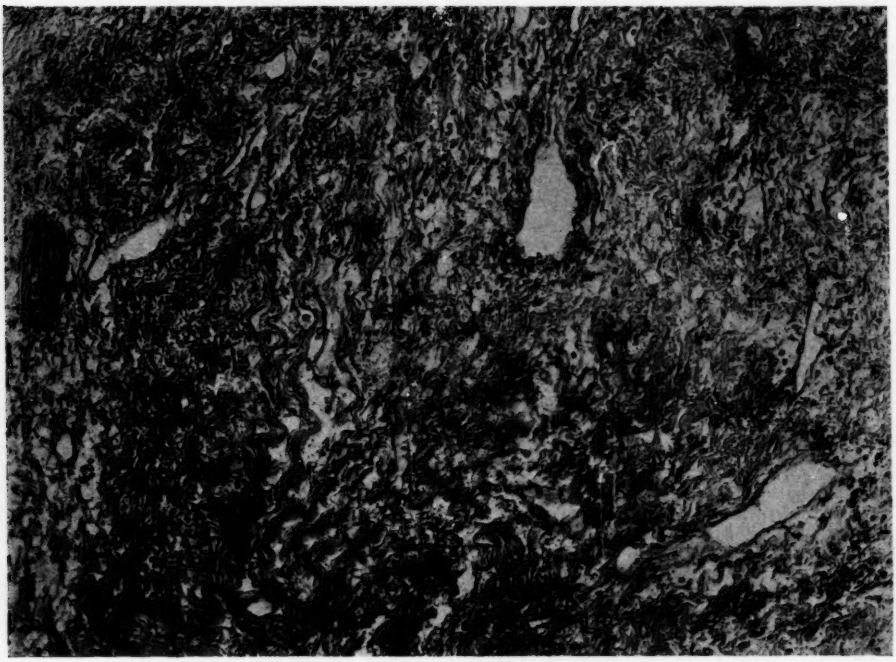

Fig. 12.—Detail of Fig. 11 (Group 4). Showing generalized edema, degeneration, and necrosis of connective tissue. Hematoxylin and eosin. Orig. mag: 175×.

DISCUSSION

The findings indicate that the oxygen consumption of human gingiva is measurable by the Warburg manometric technic. Although the number of cases does not lend itself to full statistical analysis, it appears that in gingival disease variations in oxygen consumption may be related to microscopic pathological changes.

Because all the operations in this experiment were performed under Novocain anesthesia, Novocain, 2 per cent; Adrenalin, 1-20,000, the effect of these substances upon oxygen consumption must be considered here. The literature[8-11] indicates that both these substances depress oxygen consumption. Some qualification of the QO_2 levels for human gingiva obtained in this study is

therefore indicated; however, the exact degree to which the oxygen consumption of gingival tissue is modified by Novocain anesthesia is yet to be determined.

The normal QO_2 of 1.6 ± .37 obtained in this study for normal gingiva is comparable to that reported for human skin[1, 12] (1.48 ± .48; 2.1 with variations between 1.0 and 3.6) and lower than that of liver[2] (guinea pig, 4.04) and kidney[2] (guinea pig, 13.0). The similarity in the oxygen consumption of human gingiva and skin and the differences in oxygen consumption between gingiva and liver and kidney might be anticipated by a comparison of the functional activities of the respective tissues.

The tendency toward increased oxygen consumption in marked chronic marginal gingivitis is apparently associated with an infiltration of inflammatory cells, the formation of new blood vessels and connective tissue fibers, and the proliferation of connective tissue and epithelium.

Although the findings in Table II, Group 3, indicate a trend toward increased oxygen consumption associated with the above cellular changes, an elevation in consumption was not seen in all cases. This variation in the individual oxygen consumption values in marked chronic inflammation may be attributed to the method of grouping the tissues in the experiment. It was pointed out above that the specimens were grouped according to the predominant microscopic change. In many of the sections in which there was a predominance of inflammation and proliferation, there were also some degenerative changes. Since it has been shown above that degeneration tends to lower oxygen consumption, it is likely that the presence of some degenerative changes in specimens, in which inflammatory and proliferative changes predominated, lowered the QO_2 values obtained in isolated cases in Group 3. Although the arbitrary grouping of specimens according to predominant histological changes may have introduced a factor which caused a variation in the individual QO_2 values in Group 3, it was not sufficient to negate the upward trend which characterized the group.

It is of interest to note that the increased oxygen consumption associated with cellular activity in chronic inflammation in human gingiva is comparable to the QO_2 values reported with similar microscopic changes in other tissues (kidney,[3] tendon,[4] and skin[5]).

CONCLUSION

1. The oxygen consumption of normal human gingiva is measurable by the Warburg manometric technic.

2. In gingival disease, an apparent relationship between oxygen consumption and microscopic pathological changes has been discussed.

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